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Interactions between p300 and Multiple NF-Y Trimers Govern Cyclin B2 Promoter Function*

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The CCAAT box is one of the most common elements in eukaryotic promoters and is activated by NF-Y, a conserved trimeric transcription factor with histone-like subunits. Usually one CCAAT element is present in promoters at positions between −60 and −100, but an emerging class of promoters harbor multiple NF-Y sites. In the triple CCAAT-containing cyclin B2 cell-cycle promoter, all CCAAT boxes, independently from their NF-Y affinities, are important for function. We investigated the relationships between NF-Y and p300. Chromatin immunoprecipitation analysis found that NF-Y and p300 are bound to the cyclin B2 promoter *in vivo* and that their binding is regulated during the cell cycle, positively correlating with promoter function. Cotransfection experiments determined that the coactivator acts on all CCAAT boxes and requires a precise spacing between the three elements. We established the order of *in vitro* binding of the three NF-Y complexes and find decreasing affinities from the most distal Y1 to the proximal Y3 site. Binding of two or three NF-Y trimers with or without p300 is not cooperative, but association with the Y1 and Y2 sites is extremely stable. p300 favors the binding of NF-Y to the weak Y3 proximal site, provided that a correct distance between the three CCAAT is respected. Our data indicate that the precise spacing of multiple CCAAT boxes is crucial for coactivator function. Transient association to a weak site might be a point of regulation during the cell cycle and a general theme of multiple CCAAT box promoters.

The CCAAT box is a widespread promoter element that is present in many, if not most eukaryotic promoters (1); in the vast majority of promoters, it is located in either orientation between −60 and −100 from the transcription start site (2). The importance of this element has been outlined in innumerable functional assays, and indeed, mutations affecting it have a profound negative effect on the function of neighboring cis-acting elements. Electrophoretic mobility shift assays (EMSAs)[1] and transfections with highly diagnostic dominant negative vectors for NF-YA implicated NF-Y as the CCAAT activator in >500 mammalian promoters (2).[2] NF-Y is composed of three subunits, NF-YA, NF-YB and NF-YC, all necessary for DNA binding (3). NF-YA and NF-YC possess large Q-rich domains that are required for transcriptional activation. NF-YB and NF-YC are tightly bound through histone-fold motifs, whose formation is required for NF-YA association and sequence-specific DNA binding. Circular permutation assays have clearly shown that NF-Y bends DNA and organizes the three-dimensional architecture of promoters; it is understood that NF-Y promotes the binding of neighboring trans-activators and makes connections with TFIID, contacting several of the TAF_{II}s (4). Moreover, NF-Y is able to interface with a well positioned nucleosome on the major histocompatibility complex class II Ea promoter and with a chromatin-reconstituted topoisomerase IIα promoter (5, 6).

Coactivators are a large and heterogeneous family of non-DNA-binding proteins that make use of the platforms represented by DNA binding factors to access promoters. In general, coactivators are thought to serve as a bridge for transcription factors and holoenzyme interactions, having been recruited to promoters through activation domains. Many coactivators, such as CBP/p300, PCAF, GCN5, possess an enzymatic activity, histone acetyltransferase, that adds an acetyl group to lysines of the N-terminal ends of the core histones (7). The enzymatic activity is apparently essential for activation function in many, but not in all systems tested (8–10). In addition to histones, coactivators also target transcription factors, influencing different aspects of their functions, such as DNA binding affinity, nuclear localization, or retention (for review, see Ref. 11). The role of p300/CBP in control of cell growth and differentiation has been studied in many systems (12). p300/CBP exerts a profound effect on cell cycle control, as exemplified by the finding that E1A mutants that cannot bind to p300 exhibit defective cellular transformation (13). The p300/CBP·PCAF protein complex might regulate target genes that are involved in controlling the G_1/S transition, such as $p21^{WAF1}$ (14). The overexpression of E1A, whose binding to p300/CBP antagonizes PCAF association, drives cells into S phase (15). $p300^{-/-}$ and $cbp^{-/-}$ knockout mice have provided evidence that p300/CBP proteins are important for cell cycle regulation and differentiation (16).

Because NF-Y is important for the activation of many promoters, it was natural that connections between this factor and coactivators emerged. Affinity columns identified interactions

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[1] The abbreviations used are: EMSA, electrophoretic mobility shift assay; CBP, cAMP-response element-binding protein (CREB)-binding protein; wt, wild type; CMV, cytomegalovirus; ChIP, chromatin immunoprecipitation.

[2] F. Romani and R. Mantovani, unpublished information.

of the histone-fold motif subunits with human GCN5 (17); NF-Y-PCAF interactions mediate the potent activation of the multi-drug resistance-1 promoter upon treatment of human cells with trichostatin A, and binding to p300 activates the HSP70 promoter in *Xenopus* oocytes in the absence of heat shock or the addition of heat shock factor-1 (18, 19). Among the many genes activated by NF-Y, an emerging class is represented by those regulated differently during the cell cycle, particularly in the G_2/M phase. Key regulators such as CDC25A/B/C, cyclin B1/B2, Cdc2, and topoisomerase IIα contain multiple CCAAT boxes in their promoters, invariably shown to be crucial for the proper regulation of these genes (Ref. 2 and references therein; Refs. 20–26).

Cyclin B is a central regulator for progression from G_2 to mitosis. It associates with the Cdc2 cyclin-dependent kinase 1 and thereby regulates phosphorylation of target proteins (27). Complex formation between cyclin B and Cdc2 is essential for G_2/M transition. In mammalian cells cyclin B exists in two isoforms, cyclin B1 and cyclin B2 (28). We have previously analyzed expression from the cyclin B2 promoter and found NF-Y to be the major activator (21). Cell cycle-dependent transcription is mediated by a CDE-CHR repressor element (29). Three NF-Y binding CCAAT boxes in the proximal promoter spaced 33-bp apart are responsible for more than two-thirds of the total activity of the cyclin B2 promoter. A large difference in affinity to NF-Y was detected among these three CCAAT elements, with the proximal Y3 binding NF-Y with very low affinity, yet they all equally contributed to the full transcriptional activity of the promoter (21). This raised the possibility that NF-Y binding is cooperative, either directly, through interactions with intermediate factors such as coactivators, or else that another factor, for which there is no evidence at the moment, might bind and activate Y3. To solve this matter, we present data that dissect the interplay between NF-Y and p300.

MATERIALS AND METHODS

Analysis of Mutants with Changed Spacing between CCAAT Boxes—Plasmids derived from the firefly luciferase-expressing wild type cyclin B2 promoter construct B2-Luc (21, 29) were created by PCR-based targeted mutagenesis yielding constructs with insertions or deletions between different CCAAT boxes: Y1–2Plus, **CCAAT**CAACGTGCAGAAAGGCTCGAGCTTCCAGTCTAG**CCAAT**GGGTTGCGCGCGCCCTGCGTGCGTCTAC**CCAAT**; Y1–2Del, **CCAAT**CAACGTGC↓-TCCAGTCTAG**CCAAT**GGGTTGCGCGCGCCCTGCGTGCGTCTAC**CCAAT**; Y2–3Plus, **CCAAT**CAACGTGCAGAAAGGCCTTCCAGTCTAG**CCAAT**GGGTTGCGCGCGCCCTGCTCGAGGTGCGTCTAC**CCAAT**; Y2–3Del, **CCAAT**CAACGTGCAGAAAGGCCTTCCAGTCTAG**CCAAT**GGGTTGCGCGCGC↓TCTAC**CCAAT** (insertions are shown in italics, deletions were indicated by a ↓). All plasmids were purified with anion exchange columns (Qiagen). Mutants were confirmed by DNA sequencing of both strands.

Transfections and luciferase activity assays to compare wild type with the above mutant reporters were done by lipofection with FuGENE 6 (Roche Molecular Biochemicals) as previously described (21). NIH3T3 cells (AC 59; Deutsche Sammlung von Mikroorganismen und Zellkultur, Braunschweig, Braunschweig, Germany) were transfected with 1 μg of B2-Luci wt or mutant plasmids and with 0.02 μg of pRL-null vector (Promega). Transfection efficiencies were normalized by the pRL-null cotransfection and by using *Renilla* luciferase expression assayed with the dual luciferase system (Promega). The activities of promoter mutants represent averages of nine assays, which were standardized as described (21). SaOS-2 cells (ACC 243, from DSMZ) were cultured as previously described (30). The CMV-p300 expression plasmid was generously provided by Antonio Giordano. Cells were transfected using 0.8 μg of B2-Luci wt or mutant reporter with 0.016 μg of pRL-null and 2.4 μg of CMV-p300 or pcDNA3.1 His C (Invitrogen) plasmids to keep the total DNA amount constant. Transfections and *Renilla* luciferase standardization were done as described above.

Protein Purification, EMSAs, and Footprinting—NF-Y subunits, wt and mutants, were produced in *Escherichia coli* and purified on nickel nitrilotriacetic acid columns (Sigma) as in Liberati *et al.* (30). His-tagged p300 was produced in Sf9 cells and purified through nickel nitrilotriacetic acid-agarose affinity columns. ^{32}P-Labeled fragments containing the core cyclin B2 promoter (−129 to +47) obtained by PCR were incubated under conditions described in Bolognese *et al.* (21). In the EMSA shown in Fig. 3*B*, we used a monoclonal anti-p300 antibody (purified mouse anti-p300 14991A was from Pharmingen). For footprinting assays, the wt and distance mutant cyclin B2 triple CCAAT fragments were incubated with NF-Y alone or in combination with p300 (200–400 ng) under the same conditions as in the EMSAs. After the addition of 5 mM $CaCl_2$, samples were treated with DNase I, extracted with phenol/ether, precipitated, and analyzed.

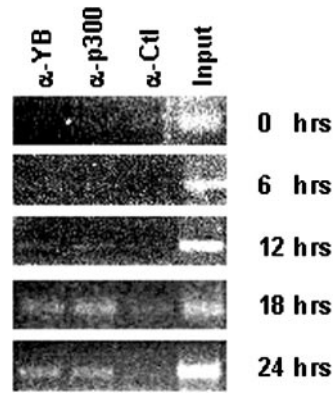

FIG. 1. **Binding of p300 and NF-Y to the cyclin B2 promoter *in vivo*.** Chromatin immunoprecipitations were performed with NIH3T3 mouse fibroblasts using the indicated antibodies. NIH3T3 serum-starved cells (G_0) were used as well as cells at 6, 12, 18, and 24 h post-restimulation by the addition of 10% fetal calf serum. *Ctl*, control.

Chromatin Immunoprecipitations—Formaldehyde cross-linking and chromatin immunoprecipitation were performed as described in Wells *et al.* (31), with the following modifications. NIH3T3 cells, serum-starved for 48 h and restimulated for 6, 12, 18, and 24 h, were fixed for 10 min with 1% formaldehyde; after quenching the reaction with 0.1 M glycine, the cross-linked material was sonicated to 500/800-bp fragments. Immunoprecipitations were performed with protein G-Sepharose (Kirkegaard & Perry Laboratories, Inc.) and 3 μg of the YB-purified rabbit polyclonals, anti-p300 (Santa Cruz SC-585X) and anti-LBP1 control. The chromatin solution was precleared by adding protein G-Sepharose for 2 h at 4 °C, separated into aliquots, and incubated with the antibodies overnight at 4 °C with mild shaking. Before use, protein G-Sepharose was blocked twice with 1 μg/μl salmon sperm DNA sheared at 500-bp lengths and 1 μg/μl bovine serum albumin, first for 2 h at 4 °C and then overnight. PCR amplifications were performed with the following primers: cyclin B2 coding, 5′-TGTAGACAAGGAAACAACAAAGCCTGGTGGCC, and noncoding, 5′ CAGCCACTCCGGTCTGCGACA.

RESULTS

p300 Is Bound to the Cyclin B2 Promoter in Vivo—The chromatin immunoprecipitation (ChIP) technique is a valuable system to detect the binding of transcription factors and coactivators to promoter sequences. We immunoprecipitated chromatin derived from exponentially growing NIH3T3 cells with anti-NF-YB, anti-NF-YC, and anti-p300, and PCR amplifications with cyclin B2-specific oligonucleotides detected the target promoter in the NF-Y immunoprecipitations (not shown); this is expected from the previously detected *in vitro* binding of NF-Y to the CCAAT boxes and activity of the NF-YA dominant negative vector (21). Next we wished to determine the association of NF-Y and p300 to the cyclin B2 promoter during the cell cycle. NIH3T3 cells were serum-starved and restimulated. Chromatin was prepared at different time points from these cells, and ChIPs were performed. Fig. 1 shows that G_0 cells have no NF-Y or p300 on the promoter. The two proteins do not associate with the cyclin B2 promoter until after 18 h of restimulation, when most cells are completing S phase and cyclin B2 expression starts. At 24 h, where most cells are in G_2/M, NF-Y and p300 are still largely bound. As a control for earlier time points, we checked a promoter, JunB, which is rapidly

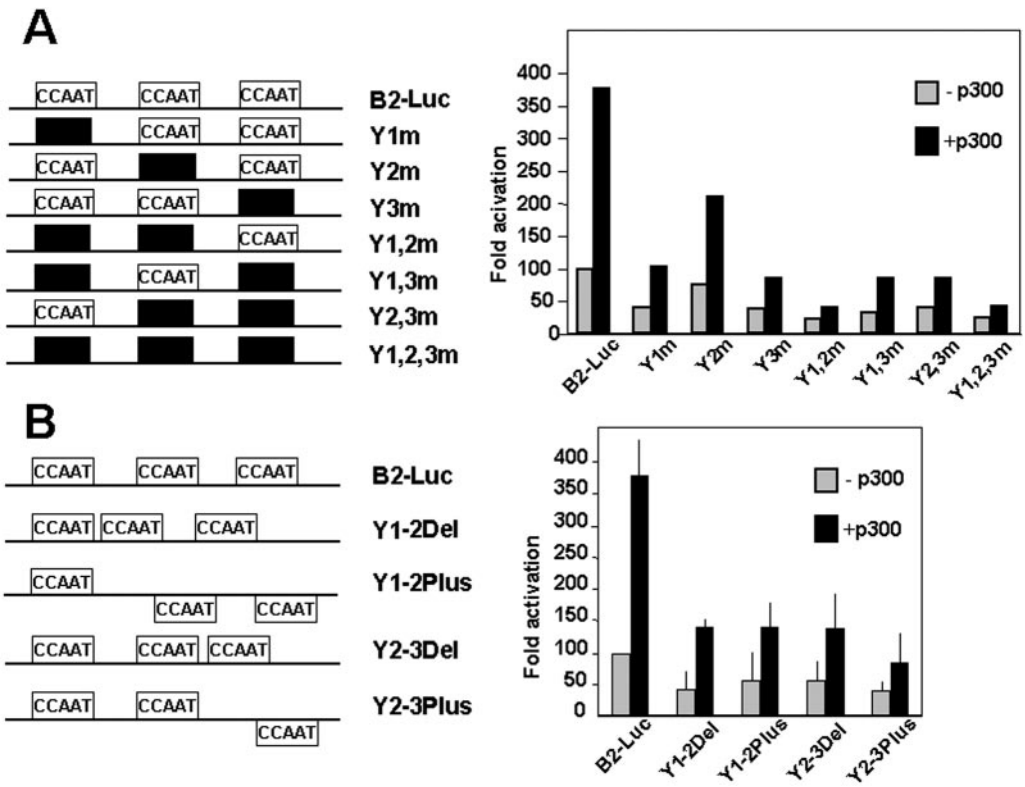

FIG. 2. **Activation of cyclin B2 by p300.** *A*, the schemes of the single, double, and triple CCAAT mutants are represented on the *left*, which are the results from reporter assays after transfection in NIH3T3 cells. *B*, equivalent to the *upper panel* with distance mutants between the three CCAAT boxes. Standard deviations were lower than 20%.

induced by serum, and found NF-Y and p300 bound in G_0 cells (Data not shown). We conclude that NF-Y and p300 association to the cyclin B2 promoter is regulated during the cell cycle, positively correlating with transcriptional activation of the gene.

Transcriptional Activation of the Cyclin B2 Promoter by p300—Because of the p300 interactions with the cyclin B2 promoter observed in ChIP assays *in vivo*, we decided to investigate their role in the transcriptional regulation of this gene. We cotransfected a p300 expression vector and the cyclin B2-luciferase reporter (21) in NIH3T3 cells. In reporter assays we observed a reproducible, although not spectacular, activation of transcription (Fig. 2*A*) that is well in line with numerous reports of similar experiments performed in other promoter systems (8–10). To verify the CCAAT dependence of the p300 activation, we used several mutant constructs with alterations in the Y1, Y2, and/or Y3 pentanucleotides described previously (21): Fig. 2*A* shows that compared with the wt cyclin *B2*, all mutant constructs are variously crippled in transcription and, most importantly, in p300 activation. In particular, mutation of the Y1/Y2 high affinity NF-Y sites and change of the all three Y1–3 boxes are essentially refractory to the activity of p300 overexpression. Additionally, mutation of Y3, which is a poor NF-Y binding site, is also severely down in p300-stimulated transcription.

It is well known that correct spacing between different transcription factors is a crucial aspect in promoter proficiency. The three CCAAT boxes of the cyclin B2 promoter are aligned, with a conserved spacing of 33 base pairs between them. Because the presence of all three CCAAT boxes is important for optimal transcription and for p300 activation, we figured that cyclin B2 transcriptional regulation represents a good system to study the role of activator alignment for p300 function. The distance between the three CCAAT sites is 33 base pairs, which represents an almost perfect alignment of the three binding elements on the same side of the double helix. We derived a set of mutants in which the spacing between Y1/Y2 and Y2/Y3 was altered, either by eliminating 10 base pairs, 1 turn of the double helix, or adding 5 base pairs. In the first case one expects to be at the limit of simultaneous NF-Y/CCAAT binding, based on experiments performed on the double CCAAT boxes of the human γ-globin promoter (32). In the second, the two NF-Y sites would be positioned on opposite sides of the DNA helix (see the scheme in Fig. 2*B*). These mutants were tested in the cotransfection assays used above with and without p300 overexpression. Fig. 2*B* shows that all distance mutants exhibited decreased activity, about 3/4-fold lower than wt, even without p300 cotransfection. More importantly, p300-dependent activation was reduced by about 2/3-fold but not completely abolished. Interestingly, the most affected mutant was Y2/Y3Plus, which is expected to affect the interactions between the two lowest affinity NF-Y sites. Overall, these data indicate that p300 activates cyclin B2 transcription by acting on intact and correctly spaced CCAAT boxes. Despite their differences in relative NF-Y affinities, all three CCAAT elements are important for p300-dependent activation.

Association of p300 to Multiple NF-Y/CCAAT Complexes—The results obtained with *in vivo* assays prompted us to set up an *in vitro* system with recombinant proteins to dissect the interplay between p300 and the NF-Y binding to the three CCAAT boxes in the cyclin B2 promoter. Recombinant NF-Y proteins were purified from *E. coli*, and p300 was purified from baculovirus. These reagents were employed in EMSAs. It should be noted that NF-Y-p300 direct interactions in solution were described in *Xenopus* (18), but we found no evidence of such interactions under our immunoprecipitation conditions.[3]

[3] G. Caretti and R. Mantovani, manuscript in preparation.

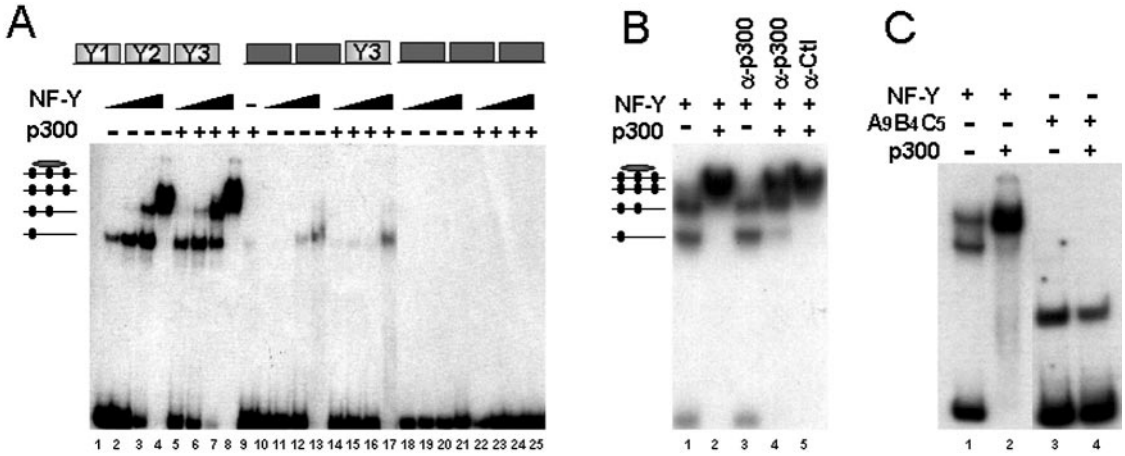

FIG. 3. **Binding of NF-Y and p300 to the triple CCAAT cyclin B2-promoter.** *A*, EMSA analysis of the wt cyclin B2 region (*lanes 1–8*), the Y1-Y2m (*lanes 9–17*), and triple CCAAT mutant (*lanes 18–25*) with increasing doses of NF-Y (0.3 ng: *lanes 1*, *5*, *10*, *14*, *18*, *22*; 1 ng: *lanes 2*, *6*, *11*, *15*, *19*, *23*; 3 ng: *lanes 3*, *7*, *12*, *16*, *20*, *24*; 10 ng: *lanes 4*, *8*, *13*, *17*, *21*, *25*) in the absence (*lanes 1–4*, *10–13*, *18–21*) or presence (*lanes 5–8*, *14–17*, *22–25*) of 100 ng of p300. In *lane 9* p300 alone was incubated with DNA. *B*, EMSA supershift. Same as *A* in *lanes 4* and *8*, except that we added anti-p300 (500 ng in *lanes 3* and *4*) or anti Gata1 (same amount in *lanes 5*) for 30 min before the addition of p300. *Ctl*, control. *C*, EMSA of wt NF-Y trimer (*lanes 3–4*) or mutants YA9/YB4/YC5 (30) were incubated with wt cyclin B2 DNA in the absence (*lanes 1* and *3*) or presence of p300 (*lanes 2–4*).

Initially, we wished to test the possibility that p300 directly binds to DNA-bound NF-Y. A cyclin B2 fragment of 200 base pairs containing the three Y boxes was labeled and used with increasing amounts of NF-Y in the absence or presence of 20 ng of p300 (Fig. 3*A*, *lanes 1–4* and *5–8*, respectively). As expected, multiple bands are generated with NF-Y, corresponding to the formation of complexes with one, two, or three trimers bound to the Y boxes (see below). Two types of information were manifest from this experiment. (i) At high NF-Y concentrations a slow migrating complex is observed only in the presence of p300 (compare *lanes 4* and *8*); (ii) at low NF-Y concentrations the addition of p300 induces an increase of double CCAAT binding (compare *lanes 2* and *3* with *lanes 6* and *7*). In parallel, the same amount of proteins was incubated with probes of identical lengths containing mutations in either Y1 and Y2 (Fig. 3*A*, *lanes 9–17*) or all three CCAAT boxes (Fig. 3*A*, *lanes 17–24*). With the Y1/Y2m probe a single band was generated only at high NF-Y concentrations, representing a single NF-Y bound to the low affinity Y3. This complex is not affected by the addition of p300. With the Y1/Y2/Y3m probe, no interaction was observed in the presence or absence of p300 even at high NF-Y concentrations. To verify the effect of p300 addition, we performed supershift EMSAs; as shown in Fig. 3*B*, formation of the upper, slow migrating complex was partially inhibited by the addition of anti-p300 antibodies, with the appearance of the non-p300 complexed NF-Y single and double complexes (compare *lane 4* with *lane 2*). A the same time, the addition of anti-p300 had no effect on the NF-Y complexes, much like an irrelevant anti-Gata antibody on the NF-Y·p300 complexes (Fig. 3*B*, *lanes 3* and *5*). Having detected a complex of discrete mobility between NF-Y and p300 on the wt cyclin B2 probe, we used a recombinant NF-Y trimer deleted of domains outside of the homology domains of NF-YA/B/C: Fig. 3*C* shows that unlike wt NF-Y, which can be bound by p300, the YA9-YB4-YC5 mutant (33, 34) is incapable of doing so.

Taken together these results indicate that a complex can be formed *in vitro* between NF-Y and p300 provided that (i) multiple CCAAT boxes are present and bind the bridging NF-Y, and (ii) domains outside the histone-fold motifs of NF-YB-NF-YC and the conserved part of NF-YA are present. Binding of p300 apparently favors NF-Y-DNA interactions.

Lack of Cooperativity of the NF-Y·p300 Complex—Our analysis on the double CCAAT box of the γ-globin promoter suggested that if a certain spacing is respected (32 bp), then cooperative binding of NF-Y molecules is possible, mainly thanks to the presence of Q-rich regions of NF-YA and NF-YC. In other systems, NF-Y is capable of improving the DNA binding affinity of neighboring factors, forming extremely stable DNA-protein complexes (3). Because of the presence of a 33-bp spacing between all three Y boxes, we considered the possibility that three NF-Ys could bind DNA cooperatively; alternatively, cooperativity could be mediated by the association of the coactivator. To investigate this point, we performed off-rate EMSA experiments with the cyclin B2 probe used above. We incubated two different quantities of NF-Y in two sets of assays for 30 min, until the binding equilibrium was reached, then added a large −200-fold excess of an unlabeled oligonucleotide containing the high affinity CCAAT box of the major histocompatibility complex class II Ea promoter. After the indicated periods of time, an aliquot of the sample was loaded on a running polyacrylamide gel. At low doses (Fig. 4*A*, *lanes 1–5*), under conditions where only single and double CCAAT binding is observed, the upper band, corresponding to Y1-Y2 occupation (see below), was rapidly competed (compare *lanes 1–3*), whereas the single interaction only slowly decreased. This behavior is not indicative of a cooperative effect, because in the latter case, we would have observed a slower decrease of the upper complex compared with the lower, single CCAAT binding activity. At higher NF-Y amounts (Fig. 4*A*, *lanes 6–10*), the triple interaction appeared. In this case, double and single CCAAT binding was still observed at late time points (*lanes 9* and *10*), but triple binding was rapidly competed, being minimal after 15 min (compare *lanes 6–8*). Because of the persistence of NF-Y binding at relatively late points (90 min in *lanes 5* and *10*), we extended the off-rates to 4, 8, 16, and 24 h, starting with NF-Y concentrations that generate triple binding. Fig. 4*B* shows that double and single, but not triple NF-Y binding, persisted after 24 h of incubation with the competing oligonucleotide. By comparison, we performed a parallel experiment in the absence of unlabeled competitor, which showed a slower off-rate for the triple complex (Fig. 4*B*, compare *lanes 1–3* with *6–8*). These latter experiments reinforce the notion that CCAAT binding by NF-Y to high, that medium affinity sites are stunningly sta-

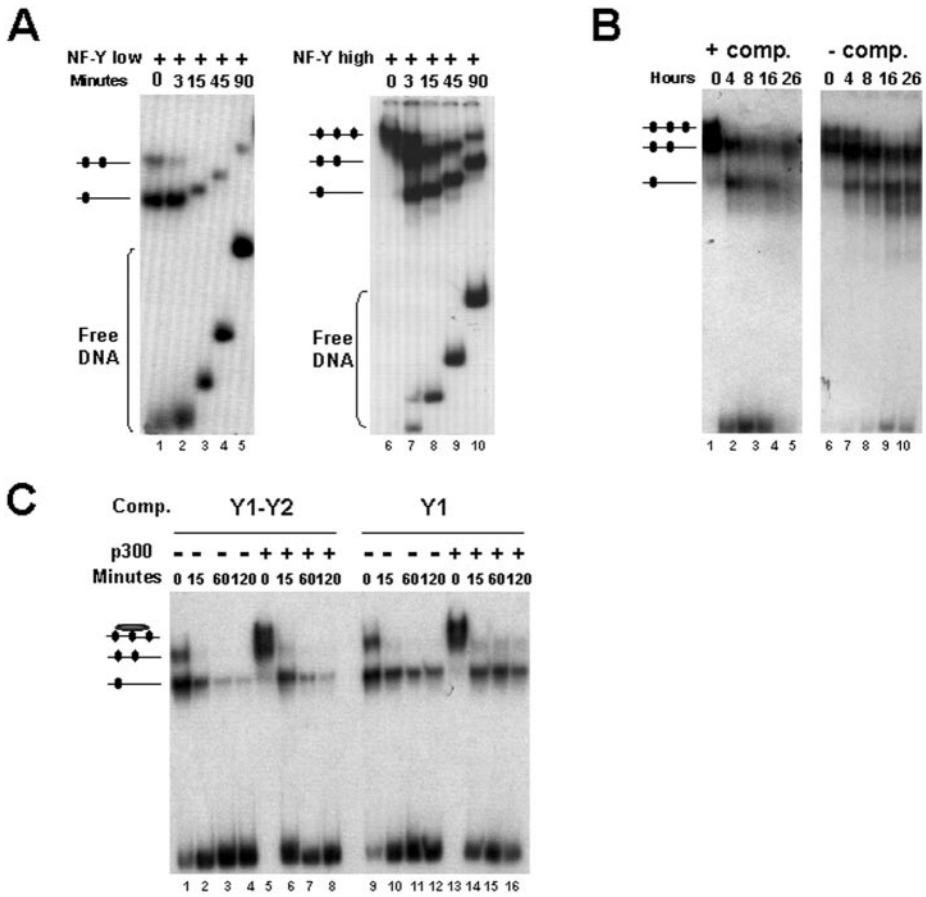

FIG. 4. **Lack of DNA binding cooperativity with NF-Y·p300 complexes.** *A*, off-rates EMSA analysis of NF-Y and NF-Y·p300 complexes on the cyclin B2 promoter. 2 ng (*lanes 1–5*) or 10 ng (*lanes 6–10*) of NF-Y were incubated with the cyclin B2 probe until equilibrium was reached and then challenged for the indicated times with 100-fold molar excess of Y1 CCAAT-oligonucleotide (21). *B*, as in *A*, except that samples with 10 ng of NF-Y were incubated for the indicated hours in the presence (*lanes 1–5*) or absence (*lanes 6–10*) of the unlabeled competitor (*comp.*). *C*, same as *A*, except that NF-Y (2 ng) was incubated in the absence (*lanes 1–4* and *9–12*) or presence (*lanes 5–8* and *13–16*) of 100 ng of p300. In *lanes 1–8* a long oligonucleotide containing both Y1 and Y2 (21) was used as a cold competitor; *lanes 9–16* used the Y1 oligonucleotide.

ble, and that no cooperativity is observed *in vitro* with either Y1-Y2 or the low affinity Y3.

One possibility to explain the transfection data of Fig. 2 could be that cooperativity is mediated by p300. We therefore performed the same type of off-rate EMSAs in the presence of p300 using as a competitor a long oligonucleotide comprising the Y1-Y2 boxes (Fig. 4*C*, *lanes 1–8*) or a Y1 oligonucleotide (Fig. 4*C*, *lanes 9–16*). Results indicate that the addition of the coactivator had no effect on the stability of the NF-Y·p300 or NF-Y triple, double, or single complexes using the Y1-Y2 competitor (compare *lanes 1–4* with *5–8*). Using the short Y1 oligonucleotide as competitor, no effect was observed on the triple complexes, whereas a modest effect is observed on the double complex (compare *lanes 13–16* with *9–12*). Most likely this is due to the less efficient competition of the single CCAAT oligonucleotide compared with the double CCAAT boxes. Collectively, these results argue against the idea that multiple NF-Ys with or without p300 are capable of forming higher order complexes that bind DNA cooperatively *in vitro*.

EMSA and Footprinting Analysis of Distance Mutants between Y Boxes—The functional data on the distance mutants shown in Fig. 2 lead us to analyze the role of spacing on NF-Y interactions with and without p300. Initially, we verified the NF-Y binding capacity in EMSA experiments with probes containing the alterations described in Fig. 2. Results in Fig 5 show that, with the exception of Y1-Y2Del, all other mutants showed normal interactions with NF-Y. The apparent difficulty in forming double CCAAT box-NF-Y complexes observed with the latter mutant (Fig. 5, compare *lane 3* with *1*) was not unexpected, because the distance between Y1 and Y2 becomes 23 base pairs, which is the lower limit for simultaneous NF-Y binding (30).

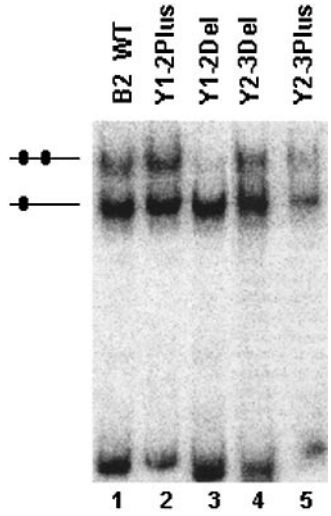

FIG. 5. **Binding of NF-Y to the CCAAT distance mutants.** EMSA analysis of NF-Y binding (2 ng) to cyclin B2 promoter mutants derived from plasmids described in Fig. 2.

Next we switched to footprinting analysis of these five probes after incubation with NF-Y and p300 (Fig. 6). *Panel A* shows that increasing concentrations of NF-Y progressively protects Y1 and Y2, but hardly Y3 (Fig. 6*A*, *lanes 1–4*). However, in the presence of p300, protections over Y2 and Y3 are observed already at lower NF-Y concentrations (Fig. 6*A*, compare *lanes 2–4* with *lanes 5–7*). Furthermore, protection of Y3 is only observed with p300 (Fig. 6*A*, compare *lanes 4* and *7*). In addition to CCAAT protections, NF-Y and NF-Y·p300 also generated hypersensitive sites at the 5′ of Y1,

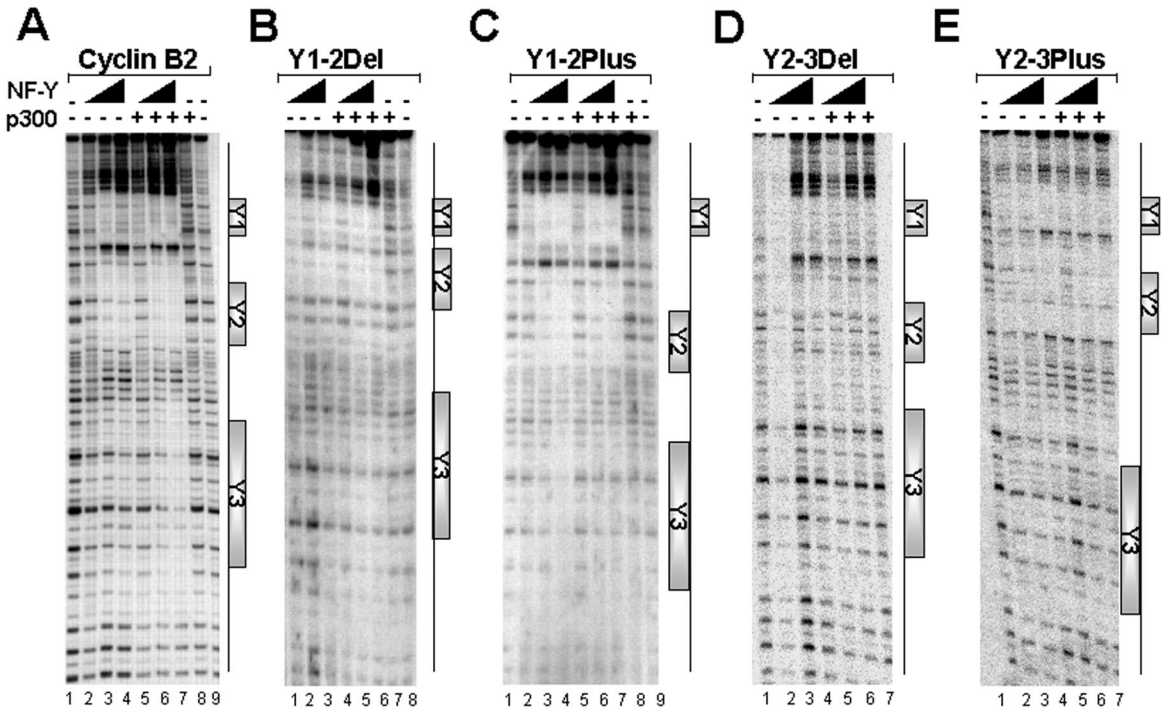

FIG. 6. **Footprinting analysis of NF-Y·p300 binding to the cyclin B2 CCAAT box distance mutants.** Increasing concentrations of NF-Y (5, 15, 50 ng) in the absence (*lanes 2–4*) or in the presence of 500 ng of p300 (*lanes 5–7*) were used with the indicated probes in *A–E*. In *lanes 1* and *9* no protein was added; *lanes 8* contained only p300.

between Y1 and Y2 and between Y2 and Y3. Note that p300 alone was incapable of DNA binding (Fig. 6*A*, compare *lanes 1* and *8* with *lane 7*). The same set of experiments was performed on the four mutant promoters; Fig. 6*B* shows the Y1-Y2Del probe, with weaker protections over Y2, no protection on Y3, and absence of hypersensitive sites between Y1-Y2 and Y2-Y3. On the other hand, the binding on Y1 is apparently normal (Fig. 6*B*, compare *lanes 1–3* and *4–6* with *lane 8*). Fig. 6*C* shows the 1–2Plus probe, which has essentially wt-like patterns of NF-Y protections and hypersensitive sites, with a decrease of the hypersensitive site between Y2-Y3 (compare *lanes 1–4* in Fig. 6*A* with *lanes 1–4* in Fig. 6*C*); the addition of p300 has negligible effects on Y1 or Y2 binding but clearly decreases Y3 interactions (Fig. 6*C*, *lanes 5–7* and *9*). With the Y2–3Del probe, binding of Y3 and to a lesser extent Y2, but not Y1, is decreased; the Y2–3 hypersensitive site is also abolished (Fig. 6*D*). Finally, with the Y2–3Plus, binding of Y1 and Y2 is normal as well as the Y1–2 hypersensitive site, but Y3 binding is negligible; the addition of p300 has essentially no effect on NF-Y binding on Y3 but has a modest effect on Y2 (Fig. 6*E*, compare *lanes 2–4* with *5–7*). Altogether, these results indicate p300 acts on NF-Y bound to Y1 and Y2 to increase the otherwise very low affinity for the Y3 CCAAT; alterations of the correct spacing between the Y1-Y2 or Y2-Y3 CCAAT boxes abolish the p300-facilitation effect.

DISCUSSION

In this study we investigated the relationship between NF-Y and p300 on the cyclin B2 promoter that is active in the G_2/M phase of the cell cycle. We found that p300 activates in a manner that requires a precise spacing between the three CCAAT boxes. Indeed, NF-Y and the coactivator are bound to the promoter *in vivo* in a cell cycle-dependent way. *In vitro* the order of binding of three NF-Y protein complexes is Y1 > Y2 ≫ Y3, with decreasing affinities from the most distal to the proximal site. The binding of two or three NF-Y molecules with or without p300 is not cooperative. However, p300 favors the association of NF-Y to the proximal site, and the distance between the three CCAAT boxes is crucial for this activity. We conclude that the precise alignment of multiple CCAAT boxes is crucial for coactivator function.

NF-Y Binding to Multiple CCAAT Boxes—A recent analysis of 1031 human promoters indicated that the CCAAT box is present in 65% of them (1). We have catalogued >500 CCAAT promoters regulated by NF-Y and found that the majority contain only one CCAAT box, either in the forward or reverse orientation. In general, NF-Y cooperates with neighboring factors to regulate gene expression. In inducible systems, such as those of the heat shock, endoplasmic reticulum stress response, and genes involved in cholesterol metabolism, NF-Y teams up with heat shock factor, ATF6, and sterol regulatory element binding protein. In the major histocompatibility complex class II genes setting, NF-Y cooperates with the regulatory factor X box trimer (Ref. 3 and references therein; Refs. 18 and 34). The mechanism of the cooperative effect has been studied and, baring the NF-Y-C/EBP connections on the albumin promoter, in all other cases NF-Y mediates a profound increase in DNA-binding affinity of the neighboring factor.

Alterations in the spacing between the CCAAT boxes and nearby sites provoke a decrease and sometimes abolition of the activation potential. In cell cycle-regulated promoters, NF-Y is essential for the timing of activation (35, 36) and repression (37, 38). In the case of G_1/S promoters, NF-Y cooperates with E2Fs; in G_2/M promoters NF-Y binding CCAAT boxes are found near the CDE-CHR element (29, 37–39). Biochemically, it is unclear at what level the cooperation is exerted. Many cell cycle promoters are peculiar in that they contain at least two and sometimes more NF-Y binding sites, in particular in promoters of key G_2/M cell cycle regulators Cdc2, topoisomerase IIα, CDC25C, and cyclin B1/B2 (Table I). The distance between these elements is (i) highly conserved among species and (ii) relatively constant; in the case of CDC25C, three CCAAT boxes are spaced 32 bp apart, in cyclin B2 the distances are 33 bp, in

TABLE I

List of cell cycle promoters with multiple CCAAT boxes spaced by 31/33 nucleotides

Sequences from cyclin B2 were from Bolognese *et al.* (21), CDC25C and Cdc2 were from Haugwitz *et al.* (32) and Zwicher *et al.* (38, 39), cyclin B1 was from Farina *et al.* (22), thymidine kinase was from Arcot *et al.* (48), and RRR2 was from Park and Levine (49).

CycB2	Mm	-79	AAGCCAG**CCAAT**CAACGTGCAGAAAGGCCTTCCAGTCTAG**CCAAT**GGGTTGCGCGCGCCCTGCGTGCGTCTAC**CCAAT**AGTGCG
CDC25C	Hs	-20	CCAGTAA**CCTAT**CCCCGCTCGCCTCTAAGCTGCGTCAG**CCAAT**CTCCGCGCGCGCGCCCAGGGCCTCATGG**CCTAT**CGTTGGGC
	Mm		TCAACAA**CCTAT**CACCACTCTCCTGCTAGCAGCGTCAG**CCAAT**CATCGCACCAGAACATGACC
CycB1	Hs	-20	GCAGCCG**CCAAT**GGGAAGGGAGTGAGTGCCACGAACAGG**CCAAT**AAGGAGGGAGCAGTGCGGGG
Cdc2	Hs	-36	GGATTCA**CCAAT**CGGGTAGCCCGTAGACTTTCAAAGCAG**CCAAT**CAGAGCCCAGCTCCGCAGTG
	Mm	-41	GGATCCG**CCAAT**CCGATTGCACGTAGACGTTCAAAGGAG**CCAAT**CAGAGCTGCTACGCTTGGGC
TK	Hs	-29	GTGCTGG**CCAAT**CACGAGCCGGCCCCGCCGCCATGGGG**CCAAT**CAGCGCCCGGCCGCTGACCT
RRR2	Hs	-123	AGCGCAG**CCAAT**GGGAAGGGTCGGAGGCATGCACAG**CCAAT**GGGAAGGGCCGGGGCACCAAAG**CCAAT**GGGAAGGGCCGGG

Cdc2 two CCAAT elements are spaced by 32 bp. Promoters for which both human and mouse sequences are available show that the sequences of NF-Y binding sites as well as their distance is strictly conserved. Especially in CDC25C and cyclin B2 mouse and human promoters nucleotides in between CCAAT boxes are not conserved, contrasting the sequence identity of and distance between NF-Y binding elements (32).[4] In all the promoters mentioned above, the affinities of NF-Y for the individual CCAAT sites differ; in CDC25C one of the CCAAT boxes is not even a perfect pentanucleotide. In cyclin B2 it is apparent that between Y1 and Y3 there is a 50-fold difference in binding affinity, yet the integrity of Y3 is as important as the high affinity Y1 binding in terms of function. The cyclin B2 systems is also conspicuous for another reason; that is, the NF-Y sites are apparently sufficient on their own to activate this promoter essentially without the need of additional factors. This is unlike the other systems tested so far, in which NF-Y is unable to activate alone. In a previous study on the γ-globin promoter, we found that NF-Y binding to double CCAAT boxes spaced by 27 nucleotides was not cooperative unless an extra 5 nucleotides were added. In such a case, a complex formed by two NF-Y trimers bound to the two sites was much more stable in off-rate experiments thanks to the presence of the NF-YA and NF-YC glutamine-rich domains (30). The distance of 32 nucleotides predicts that the two CCAAT boxes are on the same side of the DNA double helix, which has an average periodicity of 10.5 and is somewhat dependent upon the sequence. Thus, we expected that the binding of NF-Y to the triple CCAAT elements of cyclin B2 could be itself cooperative in our *in vitro* EMSAs. This is not the case (Fig. 4). It is possible that the lack of cooperativity is due to the extra nucleotide present between the cyclin B2 CCAAT boxes or to the intervening sequences. Nevertheless, it is clear that the stability of the Y1-Y2·NF-Y complexes is extremely high, with a half-life of >24 h *in vitro*. Thus, one is tempted to conclude that unlike the situation when NF-Y finds another transcription factor nearby, cooperativity at the DNA binding level is not required when two or more NF-Y sites are aligned on the promoter, even in the presence of CCAAT boxes that do not conform to an optimal binding consensus. Yet, the perfect alignment of the three sites is clearly required for optimal promoter function (Fig. 2). Optimal transcription then results from additional contacts.

Bending and phasing assays revealed that NF-Y distorts and rotates DNA in a way that is reminiscent of histones bound to DNA in the nucleosomal structure (30, 39, 40). The stunningly slow off-rates observed in this study are a premiere for sequence-specific transcription factors and can only be compared with the highly stable nucleosomal structures. Indeed, two of the NF-Y subunits, NF-YB and NF-YC, have histone-like features, as predicted from amino acid alignments, *o*-phenanthroline footprinting, and structure-function analyses and, most importantly, as detailed by recent crystallographic studies (41). A spacing of 32/33 nucleotides, thereby an alignment on the same side of the double helix, is in keeping with three CCAAT boxes forming a heminucleosomal structure, as indicated by Fig. 7. The minimal platform recognized by coactivators is likely to be NF-Y binding to Y1 and Y2, with additional contacts made on Y3 once p300 has been recruited onto the promoter.

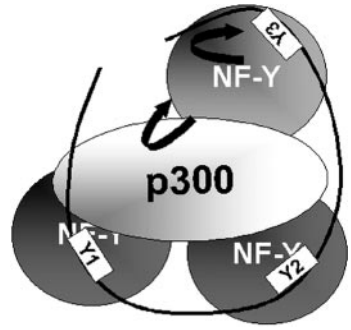

FIG. 7. **Scheme for NF-Y·p300 interactions on the cyclin B2 promoter.**

Activity of p300 on Cell Cycle Promoters—p300/CBP proteins are coactivators involved in the activation of a large number, if not all, the polymerase II-transcribed genes. In particular, p300 and CBP are targets of the adenovirus E1A oncoprotein; their role in cell cycle control has been shown by the finding that E1A mutants that cannot bind to p300 exhibit defective cellular transformation (13). The overexpression of E1A, which antagonizes PCAF binding to p300/CBP, drives cells into S phase (14). E1A is also known to affect the binding of pocket proteins to the E2F transcription factors that are associated with genes modulated in the cell cycle. Recent experiments on an E2F-regulated promoter show that p300 is important for the cell cycle-regulated expression of dihydrofolate reductase (42). The p300/CBP·PCAF protein complex is believed to regulate target genes that are involved in controlling the G_1/S transition, such as $p21^{WAF1}$ (15). Activity of p300/CBP has been studied in a number of systems, with particular focus on their

[4] M. Wasner and K. Engeland, unpublished information.

acetyltransferase enzymatic activity. We find that the positive role of p300 on NF-Y function is exerted through an increase in DNA affinity, mostly observed on the weak Y3 binding site. To the best of our knowledge, this is the first such demonstration for p300 binding on multiple sites for the same factor. Essentially three types of action are supposed to be exerted by p300/CBP; (i) the protein serves as a platform, a bridge, through which the direct interactions with multiple DNA binding activators are supported; recruitment of p300/CBP stabilizes the otherwise weak binding of these factors or even makes them possible. In keeping with this, interactions of p300/CBP with many transcription factors have been mapped in one and sometimes multiple subdomains of the coactivators. Evidence for this mechanism is still largely circumstantial, and the biochemical dissection of this mechanism on DNA was obtained only on the β-interferon promoter (43). (ii) Once on a promoter, p300/CBP proteins modify the chromatin structures nearby the sites by virtue of their histone acetylation activity, rendering nucleosomes more "accessible" to the general transcription apparatus. (iii) The same histone acetyltransferase activity would be used to increase the affinity of the DNA binding factor for the targeted sequence. This latter property is less well understood. In many cases, in fact, the opposite happens; acetylation of high mobility group(I) inhibits formation of the enhanceosome on the β-interferon promoter (43). In the case of p53, whose function is positively affected by p300, acetylation apparently affects recruitment of p300/CBP (44). Interestingly, in keeping with these latter results, we find that p300 acetylates NF-YB and that this modification increases NF-Y-p300 interactions.[3] In many promoters, the histone acetyltransferase activity of p300/CBP is apparently dispensable (8–10); p300/CBP and PCAF cooperate with members of the MyoD family of muscle transcription factors in modulating the expression of downstream myogenic factors, including myogenin and MEF2, leading to terminal withdrawal from the cell cycle of myotubes (45, 46). The p300 histone acetyltransferase domain is dispensable for MyoD-dependent transcription, suggesting that the "bridging" mechanism is predominant in this case.

One important result that stems from the ChIP analysis is that the cyclin B2 promoter is devoid of NF-Y and p300 in cells arrested in G_0, and the two activators become bound only in S-phase, when the gene starts to be activated. This was not obvious, considering the presence of NF-Y on the cyclin B1 promoter during mitosis, recently described in HeLa cells (47). Moreover, other promoters are indeed bound by NF-Y in G_0 cells, suggesting regulation of promoter selectivity in this phase. These data rule out alternative scenarios that could have been envisaged; (i) p300 would be absent from the promoter in G_1 and early S, when the promoter is silent, and be recruited in late S through interactions with a pre-bound NF-Y, constitutively associated through the cycle; (ii) it would be co-resident with the Y1-Y2 NF-Y throughout the cell cycle and only become active in late S, to promote Y3 binding. Open questions remain as to the regulation of NF-Y promoter association through the cell cycle. It is possible that additional signals, most likely post-translational modifications such as phosphorylations and acetylations, regulate this process.

A corollary to the model in Fig. 7 is represented by the possibility that NF-Y binding to Y3 is transitorily regulated by p300 during the G_2/M phase of the cell cycle; *in vivo* footprinting analysis show a much stronger protection of these two boxes in cycling cells as compared with Y3 (21). Unfortunately, the ChIP technique does not allow us to discriminate the presence of two or three molecules of NF-Y bound to cyclin B2 *in vivo*. With the present limitations of our assays it is not possible to tell whether one or more p300 molecules become associated with the promoter. Y3 is positioned just downstream of the two major start sites of this TATA-less promoter and in proximity of the CDE-CHR element recently described (29), which is also protected *in vivo*. Therefore, it is likely that it represents a key point for regulation of interactions with the proteins binding to the CDE-CHR, whose elusive biochemical nature precludes further studies at the moment. At the same time these elements may be an entry site for the general transcriptional machinery. It is also possible that the low affinity for NF-Y at Y3 has evolved by fluctuation of the sequences flanking CCAAT to create the possibility of an on-off system, less likely to happen on high affinity sites such as Y1. This type of mechanisms might not be restricted to the cyclin B2 promoter but rather a constant for cell cycle and, in general, growth-regulated promoters.

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Interactions between p300 and Multiple NF-Y Trimers Govern Cyclin B2 Promoter Function

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