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Dynamic Recruitment of NF-Y and Histone Acetyltransferases on Cell-cycle Promoters*

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Regulation of transcription during the cell-cycle is under the control of E2 factors (E2Fs), often in cooperation with nuclear factor Y (NF-Y), a histone-like CCAAT-binding trimer. NF-Y is paradigmatic of a constitutive, ubiquitous factor that pre-sets the promoter architecture for other regulatory proteins to access it. We analyzed the recruitment of NF-Y, E2F1/4/6, histone acetyltransferases, and histone deacetylase (HDAC) 1/3/4 to several cell-cycle promoters by chromatin immunoprecipitation assays in serum-starved and restimulated NIH3T3 cells. NF-Y binding is not constitutive but timely regulated in all promoters tested, being displaced when promoters are repressed. p300 association correlates with activation, and it is never found in the absence of NF-Y, whereas PCAF/hGCN5 is often found before NF-Y association. E2F4 and E2F6, together with HDACs, are bound to repressed promoters, including the G_2/M Cyclin B2. As expected, an inverse relationship between HDACs association and histones H3/H4 acetylation is observed. Blocking cells in G_1 with the cyclin-dependent kinase 2 inhibitor R-roscovitine confirms that NF-Y is bound to G_1/S but not to G_2/M promoters in G_1. These data indicate that following the release of E2Fs/HDACs, a hierarchy of PCAF-NF-Y-p300 interactions and H3-H4 acetylations are required for activation of cell-cycle promoters.

Promoters and enhancers are a combinatorial puzzle of DNA elements recognized by sequence-specific regulators that recruit coactivators and corepressors and act in a complex chromatin context. Genes that are regulated during the cell-cycle are typically active in one phase, whereas they are inactive in all others. This type of tight regulation is under the control of the E2 factors (E2Fs).[1] Originally identified for their role in the G_1/S transition, they belong to two classes, the activators (E2F1/2/3) and the repressors (E2F4/5/6) (reviewed in Ref. 1). Repression is exerted on promoters active in G_1/S under serum starvation conditions through the recruitment of negative complexes containing retinoblastoma and retinoblastoma family members, histone deacetylases (HDACs), and histone methyltransferases (2–5). Recently, genome wide approaches using chromatin immunoprecipitations (ChIP) with E2F4 and E2F1 antibodies clearly indicated that E2Fs are more general regulators of cell growth, checkpoints, and DNA repair genes as well (6, 7).

In addition to E2F sites, most cell-cycle regulated promoters, particularly those of regulators of the cycle, also contain CCAAT boxes. Genes activated in G_0 (PDGFβ-R), G_1/S (E2F1, CDC25A, and PCNA), S (Cyclin A and CDC25B), S/G_2 (topoisomerase IIα and PLK), and G_2/M (CDC25C and Cyclin B1/B2) all contain CCAAT boxes, invariably shown to be crucial for the proper regulation of these genes (8–17). *In vivo* footprinting experiments indicated that the CCAAT boxes are protected (10, 18, 19). A combination of electrophoretic mobility shift assays (EMSAs) and transfections with highly diagnostic dominant negative vectors implicated nuclear factor Y (NF-Y) as the common CCAAT activator (20). NF-Y is composed of three subunits, NF-YA, NF-YB, and NF-YC, all necessary for DNA binding. NF-YA and NF-YC possess large Q-rich activation domains. NF-YB and NF-YC dimerization is a prerequisite for NF-YA association and sequence-specific DNA binding. NF-YB and NF-YC contain histone fold motifs (HFM) found in all core histones, which mediate dimerization and DNA binding (Ref. 21 and references therein). In all systems of inducible transcription tested so far (heat shock, cholesterol biosynthesis, endoplasmic reticulum stress, and CYP genes), ChIP experiments determined that NF-Y is bound *in vivo* before gene activation (22, 23)[2]; indeed, NF-Y is bound to a transcribing Cyclin B1 promoter during mitosis in HeLa cells (24). In G_1/S promoters, NF-Y cooperates with E2Fs (8). In G_2/M promoters, NF-Y-binding CCAAT-boxes are usually found in multiple copies near the CDE-CHR elements (10–12, 15–17). These latter conserved boxes have been identified by genetic experiments as the crucial determinants of proper regulation, that is repression in G_0, G_1, and early S (19). However, biochemical characterization of the CDE-CHR interacting protein(s) has been elusive. In the case of Cdc2, circumstantial evidence suggests that E2Fs might partake in promoter regulation through the CDE-CHR (25).

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[1] The abbreviations used are: E2F, E2 factor; NF-Y, nuclear factor Y; HAT, histone acetyltransferase; HDAC, histone deacetylase; HFM, histone fold motif; ChIP, chromatin immunoprecipitation; PCAF, p300/CBP-associated factor; TAF, TBP-associated factor; PCNA, proliferating cell nuclear antigen; EMSA, electrophoretic mobility shift assay; TopoIIα, topoisomerase IIα; CBP, CREB-binding protein; PGDFβR, platelet-derived growth factor β receptor; PLK, Plo-like kinase; CDE, cell cycle-dependent element; CHR, cell cycle gene homology region; RNR-R1, ribonucleotide reductase 1; TBP, TATA-binding protein.

[2] A. Testa and R. Mantovani, manuscript in preparation.

A further level of regulation is exerted by coactivators and corepressors containing histones modifying activities, histone acetyltransferases (HATs), and HDACs. The former serves as a bridge for transcription factors and holo-enzyme interactions, having been recruited to promoters through the activation domains of the DNA-binding activators. Not surprisingly, connections between NF-Y and HAT coactivators have emerged. Interactions of the HFM subunits with hGCN5 were reported (26). p300/CBP-associated factor (PCAF) mediates the trichostatin A induction of the MDR1 promoter through NF-Y (27). Treatment with HDAC inhibitors induces the TGFβ-RII promoter by increasing the affinity of PCAF for the NF-Y trimer (28). Binding to p300 activates the *HSP70* promoter in *Xenopus* oocytes, in the absence of heat shock or addition of heat shock factor 1 (29). In this latter study, p300 was able to acetylate NF-YB *in vitro*, but the function of this modification is currently unknown. Negative regulation through NF-Y, HDAC activity, and histone deacetylation also surfaced (28, 30, 31). These latter results suggest that NF-Y might be instrumental in the establishment of repressive as well as activating complexes on the regulated promoters. We decided to use the powerful means of chromatin immunoprecipitation to gain a more complete view of the association of NF-Y, HATs, HDACs, and E2Fs to cell-cycle regulated CCAAT-containing promoters.

MATERIALS AND METHODS

Cell Line and Culture Conditions—NIH3T3 mouse fibroblasts were grown in RPMI under standard conditions with 10% fetal calf serum. Cells were arrested by incubation for 60/72 h in 0.5% fetal calf serum, and restimulation was obtained by addition of fresh medium with 10% fetal calf serum. Cell-cycle progression was monitored by DNA content through propidium iodide staining and FACScan analysis. For R-roscovitine treatment, the drug (Cyclacell, Dundee, UK) was used at a 20 μM concentration for 16 h. Under these conditions all cells were blocked in G_1, as reported in Ref. 32.

Chromatin Immunoprecipitations—Formaldehyde cross-linking and chromatin immunoprecipitation was performed as described in Ref. 3. NIH3T3 cells ($0.5/1.10^8$) were washed in phosphate-buffered saline and incubated for 10 min with 1% formaldehyde. After quenching the reaction with glycine 0.1 M, the cross-linked material was sonicated into chromatin fragments of an average length of 500/800 bp. Chromatin was kept at −80 °C. Immunoprecipitations were performed with ProtG-Sepharose (KPL) and 3–5 μg of the indicated antibodies: anti-YB purified rabbit polyclonal (23); anti-p300 (Santa Cruz, sc-585x); anti-PCAF and anti-GCN5 (a kind gift of Y. Nakatani, Harvard University, Boston, MA); anti-E2F4 (Santa Cruz, sc-1082x); anti-E2F1 and E2F6 (Active Motif, 39313 and 39509); anti-HDAC1 (Sigma, H3284); anti-HDAC 3 and HDAC4 (Active Motif, 40968 and 40969); and anti-acetylated H3 and H4 (Upstate, 06-599 and 06-866). The chromatin solution was precleared by adding ProtG-Sepharose for 2 h at 4 °C and was aliquoted and incubated with the antibodies overnight at 4 °C on a rotating wheel. ProtG-Sepharose was blocked with 1 μg/μl salmon sperm DNA (Sigma) and 1 μg/μl bovine serum albumin overnight at 4 °C and then incubated with chromatin and antibody for 2 h. Immunoprecipitated material was washed 9 times with wash buffer as described in Ref. 3. Cross-links were reversed by incubating samples for 5 h at 65 °C in 200 mM NaCl and 10 μg of RNase A to eliminate RNA. Recovered material was treated with proteinase K, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated. The pellets were resuspended in 50 μl of H_2O and analyzed by semi-quantitative PCR with *Taq*DNA Polymerase (Sigma) and the following primers: nucleolin, 5-GTGACCGGGATGGACACG-3 and 5-CCCACTTTCTCTCGCTTCCAG-3; PDGFβ-R, 5-CAGACACACGCGTCCACCCTC-3 and 5-GGGCCCGCTCTGGCTCTGGA-3; c-Jun, 5-CCAACTGTAGGAGGAGCGCAGCAGC-3 and 5-GATGAACAGTCCGGAGTCCGC-3; JunB, 5-CGCGCAGAGCCACCCGGCTC-3 and 5-GTGGTACGCCTGCTTTCTCGG-3; E2F1, 5-ATCGGAGCCTCCGTCGTCACA-3 and 5-AGGCCGCGGCGAGGGCTCGAT-3; PCNA, 5-CTTCCGTGGCGCGGAAACTTCC-3 and 5-CTGCGCGAGGTCATGACGCCA-3; RNR-R1, 5-GGTGTTCTGGATATCTGGTTGGTTA-3 and 5-GGCGGAAACTGAGCAGTAGACA-3; Cyclin A2, 5-TGTAAGATTCCCGTCGGGCCTTC-3 and 5-AGGCGGGAGGAGCGTAGAGC-3; TopoIIα, 5-TCCATTTTGAAGATTCTCCCGCCT-3 and 5-CGAGAATCCGGAAAGCGACAAAAC-3; PLK, 5-CAATCCTGAGCAAACGCAGTCCTC-3 and 5-GTTGCTCCACGAACCTGGCTGAG-3; Cyclin B2, 5-CTAGCAAGCCAGCCAATCAACGTGC-3 and 5-GGTCTGCGACACCGTTGGGAGG-3; Cdc2, 5-AGTCAGTTGGCGCCCGCCCT-3 and 5-CACACCGCAGTTCCGGACTG-3; CDC25C, 5-GGCGAGAGAATTTAGTACAAGGA-3 and 5-CTCCGGAGATGGCCTGAAGGC-3.

RESULTS

In Vivo Binding of NF-Y and HATs to CCAAT Cell-cycle Promoters—To assess the *in vivo* recruitment of NF-Y and HATs on cell-cycle promoters, we used the ChIP assay with anti-YB and anti-HATs (p300, PCAF, and GCN5) antibodies in the NIH3T3 mouse fibroblasts system (3). Chromatin from cells starved by serum withdrawal (G_0) and after restimulation with 10% fetal calf serum for 6, 12, 18, and 24 h was prepared and immunoprecipitated with anti-NF-Y, p300, PCAF, GCN5, and control anti-leader-binding protein 1/CCAAT protein 2 antibodies. Fluorescence-activated cell sorter analysis of the cell populations at the different time points was run to verify cell-cycle progression, which was indeed as expected and shown in Fig. 1. Note that the 24 h time point was more heterogeneous with 20/30% of the cells that re-entered G_1. We first amplified a control fragment in the nucleolin CCAAT-less intronic enhancer (Fig. 1, *upper panel*, and Ref. 33). The anti-NF-Y, p300 antibodies were negative, whereas CCAAT protein 2 was apparently bound between 6–18 h (33). This factor has been implicated in the activation of the CCAAT-less TS promoter in S phase (34). PCAF/GCN5 were weakly positive at 18 h, and this correlates with the binding of MYC and TRRAP, although with a slower kinetic (33).

The CCAAT promoters chosen for analysis are active in G_0 (PDGFβ-R) shortly after the G_0-G_1 transition (c-Jun and JunB), late G_1/early S (E2F1, RNR-R1, PCNA, and Cyclin A), late S/G_2 (TopoIIα, CDC25C, and PLK), and G_2/M (Cdc2 and Cyclin B2). Fig. 1 shows a comprehensive analysis of the results obtained with the target promoters in the different time points.

It is clear that NF-Y binding to these promoters is not constitutive. In G_0 cells, it is present only on PDGFβ-R, c-Jun, and JunB. At 6 h, these promoters are negative, whereas association is weakly positive to E2F1. In Late G_1, NF-Y binding is maximal on E2F1, PCNA, and RNR-R1 promoters, and detectable on TopoIIα, Cyclin A, PLK, and CDC25C. Finally, Cdc2 and Cyclin B2 become positive only at 18 h. It should also be noted that promoters that have been transcriptionally switched off, such as PDGFβ-R, cJun, and JunB at 6 h and PCNA at 24 h, are unloaded of NF-Y, whereas the occupancy of others (RNR-R1 and E2F1) is considerably decreased. We conclude that the binding of NF-Y to cell-cycle promoters is highly dynamic.

The pattern of p300 binding closely matches promoter activation. In G_0, the only promoter that is clearly positive is the transcribing PDGF-βR, but weak association is observed with c-Jun and JunB, whereas all other promoters are negative. These data correlate well with histone H4 acetylation on PDGF-βR (Fig. 3), and the low but detectable levels observed in previous studies on JunB (35). These promoters are inactive at 6 h (14, 35). G_1/S promoters are positive at 12 h, later becoming negative, and Cdc2 and Cyclin B2 are bound at 18 h. Binding to Cdc2, but not to Cyclin B2, is substantially reduced at 24 h. S/G_2 promoters are somewhat intermediate, being already associated at 12 h. It is important to note that p300 is never found on promoters in the absence of NF-Y.

The binding of PCAF to all promoters is, in general, more pronounced than that of hGCN5, with the exception of RNR-R1 at early G_1 time points. This most likely reflects the far higher (5/10-fold) amounts of PCAF in NIH3T3 cells. The patterns of binding of these HATs appear to precede promoter activation, often well before NF-Y and p300 binding. In G_0 cells, only the

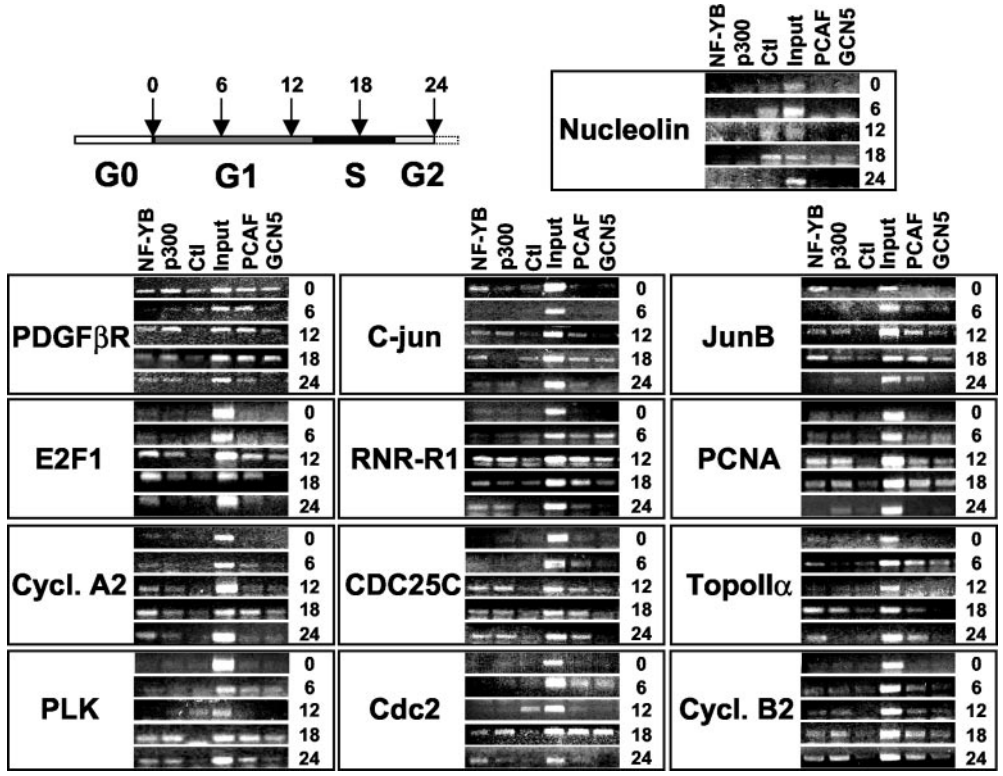

FIG. 1. ***In vivo* binding of NF-Y and HATs to CCAAT cell-cycle promoters.** Chromatin was prepared from cells at G_0 after starvation or 6, 12, 18, and 24 h after serum addition, as indicated, and immunoprecipitated with anti-NF-YB, anti-p300, anti PCAF, anti-GCN5, and anti-CCAAT protein 2 control antibodies.

transcribing PDGFβ-R is bound, whereas at 6 h all three G_1/S promoters, TopoIIα, PLK, and CDC25C, are significantly bound in conditions in which neither NF-Y nor p300 are associated. Cyclin B2 starts to be loaded at 12 h. Interestingly, PCNA, RNR-R1, E2F1, Cyclin A, and Cdc2 become rapidly unloaded of PCAF after the promoter has been switched off. In the case of PDGFβ-R, PCAF is on at 6 h before reloading of NF-Y and p300 at 12 h. As for c-Jun and JunB, which are rapidly (0.5 h) activated following serum addition, PCAF/GCN5 are not bound in G_0 nor after 6 h. Most likely, we have missed the early PCAF/hGCN5 loading because of the kinetic used in our assays; however, the two HATs are on at 12 h. Note that maximal NF-Y and p300 reloading on JunB occurs at 18 h.

In Vivo Binding of E2Fs and HDACs to CCAAT Cell-cycle Promoters—The experiments shown above prompted us to control the association of the E2F factors to CCAAT promoters. Essentially, the same protocol was used with anti-E2F1/4/6 antibodies on a representative set of promoters. Fig. 2 shows the results of such ChIP analysis. The PDGFβ-R promoter is apparently devoid of E2Fs, at least within the time points considered. The E2F1 promoter is bound by E2F4 and, to a lesser extent, by E2F1 in G_0 cells. At 12 h, when the gene is being transcribed, E2F1 clearly becomes prominent, then declines rapidly. Cdc2 and Cyclin A show a similar behavior, except that association of E2F1 in S is not observed and E2F4 is clearly bound at later time points (24 h) when a proportion of the cell population (20/30%) is re-entering G_1. Furthermore, the repressive E2F6 is bound to Cyclin A (weakly) and to Cdc2 (more strongly) in G_0 cells, rapidly becoming unloaded at 6 h. Because E2F4/1 have been detected on several S/G_2 promoters in genome-wide experiments (6, 7), we also tested Cyclin B2 in these ChIPs. It is clear that E2F4 and, to a lesser degree, E2F1, are associated in G_0, decreasing at 6 h and desappearing at 12 h. E2F6, which is not bound in G_0, is the prominent E2F bound at 6 h. Overall, this analysis confirms and extends the

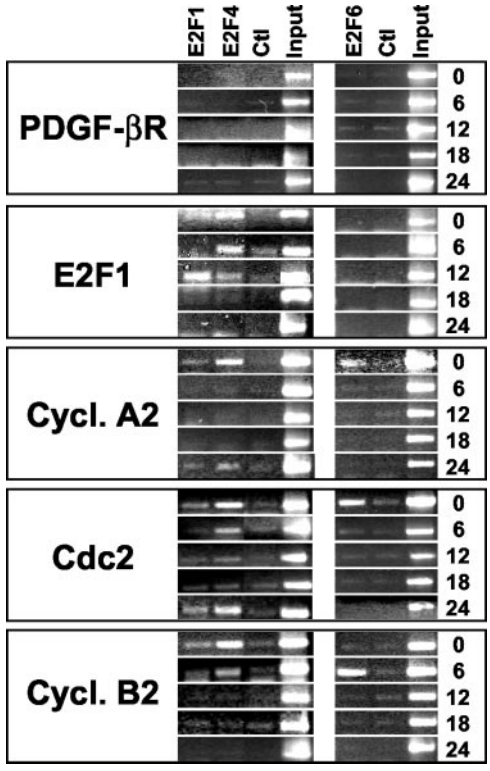

FIG. 2. ***In vivo* binding of E2F1/4/6 to a set of CCAAT promoters active in G_0 (*PDGFβ-R*), G_1/S (*E2F1*), S (*Cycl. A2*), and G_2/M (*Cdc2* and *Cycl. B2*) promoters.**

data previously reported in the NIH3T3 system (3) and in human fibroblasts (2, 4, 5), with the exception of Cyclin B2, previously undetected as an E2F target.

HDACs are associated with repressive E2Fs in many of the

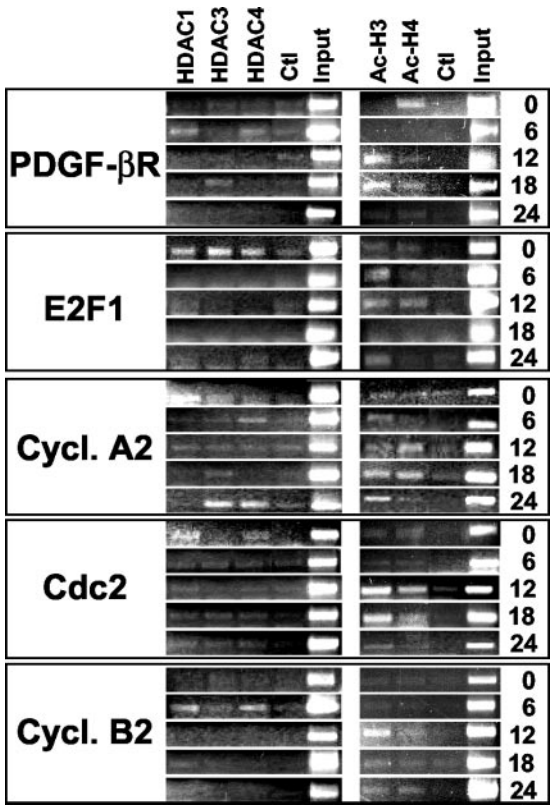

FIG. 3. ***In vivo*** **binding of HDAC1/3/4 and histones acetylation to the CCAAT promoters analyzed in Fig. 2.**

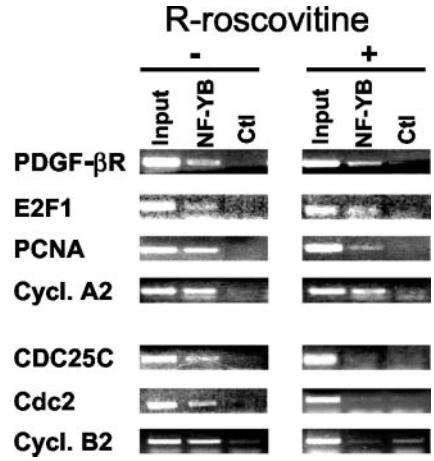

FIG. 4. ***In vivo*** **analysis of NF-Y binding to cell-cycle promoters in cycling NIH3T3 (*left Panel*) or G_1/S arrested with R-roscovitine (*right panel*).**

E2F-regulated promoters and that HDAC binding correlates with low levels of histones H3 and H4 acetylation (2, 3). Fig. 3 shows ChIP analysis with antibodies against HDAC1/3/4 and acetyl-K of histones H3 and H4. The PDGFβ-R promoter is apparently devoid of HDACs at G_0, and indeed acetylation of H4 is observed, as expected for an actively transcribing promoter. At 6 h, histones are deacetylated and HDAC1/4 are bound. At later time points histones (expecially H3) are acetylated, with little binding of HDACs. The E2F1 promoter is bound by HDAC1/3/4 and the histones deacetylated in G_0. HDACs association is no longer visible beyond 6 h, whereas H3 and, subsequently, H4 acetylation is observed during activation. Cdc2 and Cyclin A show binding of HDAC1 in G_0, which is released during activation at 12/18 h. On Cyclin A, HDAC3/4 become reassociated at the late 24 h time point. The levels of histone acetylation correlate with promoter activation, indeed preceding it and being specular to that of HDAC binding. On Cyclin B2, HDAC1/4 are associated at 6 h, consistent with the binding of E2F6 at this time point, but not with the binding of E2F4 at G_0. Acetylation of H3 is maximal at 12 h, following HDACs release and before transcriptional activation. The data presented lead us to conclude that the acetylation of histones H3-H4 tails faithfully reflects the HATs/HDACs dynamic behavior on these promoters, further validating the data on HATs and NF-Y binding.

NF-Y Is Differentially Bound in Cells Blocked at the G_1/S Transition—The results obtained above with a protocol of serum starvation-restimulation might be peculiar to a situation of G_0-G_1 re-entry and not reflect changes of promoter occupancy in normally growing cells. We sought to verify the behavior of NF-Y in a different experimental setting, that is in cells arrested in their normal cycles with the cyclin-dependent kinase 2 inhibitor R-roscovitine (32). Treatment of cycling cells with pharmacological doses of the drug indeed leads to a complete block in G_1 (not shown). Fig. 4, *left panel*, shows that the promoters of all genes are bound by NF-Y in cycling cells, a result well expected, given the heterogeneous nature of this cell population. After the R-roscovitine block, NF-Y is found only on G_1/S promoters (Fig. 4, *right panel*). In full support of the data of Fig. 1, however, in none of the G_2/M promoters NF-Y is loaded, a clear indication that the late G_1 block has prevented its association with promoters active in subsequent phases. Thus, we conclude that the cell-cycle-dependent association of NF-Y to its targets is not a peculiar phenomenon of serum restimulated fibroblasts, but rather a theme common to normally growing cells.

DISCUSSION

Through the use of the chromatin immunoprecipitation technique, we report three important results. (i) Contrary to predictions based upon *in vitro* binding activity in EMSAs and ChIP data on other promoters, the *in vivo* binding of NF-Y to cell-cycle genes is strictly regulated during the different phases of the cell-cycle. (ii) We detail for the first time the association of p300 and PCAF/GCN5 to cell-cycle promoters *in vivo*. The distinct timing of binding of these two HAT families suggests different functions. (iii) Although confirming and extending previous ChIP data on E2Fs, HDACs, and histones H3-H4 acetylation, we further detect E2F4/6 on the G_2/M Cyclin B2 promoter, thus establishing that promoters active in all phases of the cycle are regulated by E2Fs.

The bulk of the data presented here on E2F1/4/6 in Fig. 2 are in large agreement with previous experiments (2–5) performed on the Cdc2, Cyclin A, and E2F1 promoters in human systems and on Cdc2 in the mouse NIH3T3 cells. Here we find that E2F4 is also bound to Cyclin B2 in G_0 and early G_1, with the repressive E2F6 becoming the predominant E2F in this latter phase, concomitant with the binding of HDACs, histones H3-H4 deacetylation, and promoter repression. This switch among repressive E2Fs has not been described before and might be peculiar to G_2/M genes. Interestingly, E2F binding fits well with the activity of the CDE-CHR promoter elements (16 and 19 and references therein). These cis-acting sites, found in Cyclin B1/B2, Cdc2, CDC25 and Cyclin A, have been detailed with genetic experiments as required to keep promoters at bay until S phase. We propose that E2Fs are part of the elusive CDE-CHR binding and regulating activities and that S/G_2 promoters are under negative regulation in G_0-G_1 through E2F4/6 binding, with activators binding after E2Fs/HDACs repression is relieved in late G_1. In Cyclin B2, a gap in late G_1 is observed between the release of repressors and NF-Y binding, during which histones, particularly H3, are hyperacetylated (Fig. 3).

This is in agreement with the association of PCAF (Fig. 1), but it is unclear at the moment which factor(s) is bound to DNA in this phase and recruits the HAT activity. It might be one of the activating E2Fs that have not been analyzed here (E2F2/3) or a protein such as MYC, which binds *in vivo* to an E box in the related Cyclin B1 promoter (36).

The highly dynamic role of NF-Y described in this set of promoters does not match previous experiments on inducible systems, which are constitutively bound by NF-Y (22, 23). Our data on the rapidly inducible JunB and c-Jun reinforce the notion that NF-Y binding is a prerequisite for promoter activation and precedes stable p300 binding. NF-Y is generally required to set the chromatin stage in the near proximity of transcriptional start site(s), thanks to its peculiar histone fold nature, and allow further activators/coactivators buildup (20, 29). Because E2Fs regulate most, if not all, cell-cycle promoters and CCAAT boxes are often adjacent, the two proteins are likely to interplay. In the E2F1 promoter, for example, NF-Y might still be required for E2F1 association or function, as suggested by transfection studies (9), whereas E2F4 might actually prevent binding of NF-Y until late G_1. In G_2/M promoters, which contain at least two and sometimes more NF-Y binding sites, whose distance is highly conserved and constant (10–12, 15, 17), two or three NF-Y might be self-sufficient, without the need of an activating E2F. Alternatively, one of the activating E2Fs not analyzed here might partake in activation through the CDE-CHR.

One of the fundamental questions raised by our results is how NF-Y discriminates between the different targets *in vivo* in any given phase of the cell-cycle. Two important facts should be remembered: (i) the overall CCAAT-binding activity of the trimer, as assayed *in vitro* in EMSAs, is nearly equally abundant in all phases of the cycle (10), and (ii) all the binding sites analyzed here are optimal in terms of NF-Y affinity *in vitro*. What then directs NF-Y to PDGFβ-R in G_0, E2F1 in late G_1, and Cyclin B2 in G_2? A first mechanism might be promoter-driven, that is, the specific positioning of the CCAAT boxes with respect to nucleosomes influence NF-Y binding. This is suggested by *in vitro* experiments in which recombinant NF-Y easily accesses a well positioned nucleosome on the tissue-specific Major Histocompatibility Complex class II Ea promoter (37), but it is incapable of doing so on a similar nucleosome reconstituted on the Cyclin B2 promoter, despite the presence of three CCAAT boxes.[3] A second mechanism might point to specific post-translational modifications, such as the acetylation of NF-YB detected in *Xenopus* (29), which might alter promoter penetrance of the trimer. Finally, the overall acetylation status of nucleosomes could locally influence association, as suggested by our finding here that Cyclin B2 nucleosomes are maximally acetylated before NF-Y binding. These three hypotheses are most likely not mutually exclusive and pose a formidable task at dissecting the mechanistic details.

Another important aspect of our data concerns the association of HATs to cell-cycle promoters. The role of p300/CBP in the control of cell growth is exemplified by the finding that (i) E1A mutants that cannot bind to p300 exhibit defective cellular transformation; (ii) $p300^{-/-}$ and $cbp^{-/-}$ knockout mice have impairments in cell-cycle regulation and differentiation; and (iii) the HAT activity is required for G_1/S transition, by regulating E2Fs directly through acetylation and phosphorylation (38, 39, and reviewed in Ref. 40). The proteins mediating p300 recruitment might vary in a promoter-specific way. On E2F1, a combination of E2F1 and NF-Y might be required, whereas in Cyclin B2 the multiple NF-Ys might be sufficient, as suggested by *in vitro* experiments (17).

[3] G. Caretti and R. Mantovani, unpublished observations.

The association of the p300-related CBP and of PCAF/GCN5 was detailed in studies on the inducible IFN-β and on the tissue-specific α_1-AT promoters (41–43). On IFN-β, GCN5 association precedes that of CBP, which is concomitant with that of PolII and is followed by TBP and TBP-associated factors (TAFs) recruitment (41), on α_1-AT, TBP, TAFs, and PolII are on before PCAF and CBP association (43). Our findings are more reminiscent of the IFN-β system because the recruitment of PCAF/GCN5 is manifestly antecedent to that of p300. Ample evidence exists of the PCAF/GCN5 involvement in cell-cycle control (reviewed in Ref. 44). Because of the early timing of their association, it is tempting to speculate that these HATs actually help recruit NF-Y on some promoters, and not vice-versa. Unlike for p300, NF-Y has a high intrinsic affinity for PCAF/GCN5, as these HATs are found in NF-Y immunopurified fractions; the interactions are mediated by the NF-YB-NF-YC HFM subunits (26). What then recruits PCAF/GCN5? In some promoters, such as E2F1 and Cdc2, it might be one of the E2Fs, but the picture is likely to be more complex, as these HATs exist as a multi-subunit complex (also known as TFTC or STAGA) formed by 15 or more proteins, including many HFM-containing TAFs (45–47). Functional interactions between E2Fs, GCN5, and TRRAP (the largest subunit of the complex) have been described (48). It is currently unknown whether the composition of the complex actually changes *in vivo* during cell-cycle progression and whether different subcomplexes specifically target different classes of promoters. In addition to PCAF, NF-Y is able to bind *in vitro* to some of the components of the complex, such as the HFM-containing TAF12, possibly a partner of the H2A-like STAF42 (47). Other H3/H4-likes (the PAF65α-TAF9 presumed dimer) might interact with NF-Y as TAF6-TAF9 do (49). In the IFN-β system cited above, NFkB and ATF2 serve as a pre-bound platform for HATs recruitment (41), whereas in the α_1-AT promoter only Hepstatic Nuclear Factor 1 is constitutively bound, and the DNA-binding protein Hepstatic Nuclear Factor 4 becomes associated later (43). In this respect NF-Y behaves like this latter factor being associated shortly before activation. Thus, we favor a scenario in which transitory recruitment of the PCAF complex through E2Fs, presumably subsequent to the release of inhibitory components of the E2F/retinoblastoma/HDAC complexes, followed by a burst of histone acetylation, favors efficient NF-Y binding, which in turn is required for p300 association, the latter phenomenon coinciding with transcriptional activation. Finally, the displacement of NF-Y might be instrumental in turning off transcription (see PDGFβ-R, cJun, JunB, E2F1). Given the stunning stability of NF-Y-CCAAT complexes *in vitro*, we suspect that an active mechanism must be operational to remove it *in vivo*. This might involve modifications of the protein of nearby nucleosomes or the activity of chromatin remodellers. It will now be interesting to assess the association of other subunits of the PCAF complex, of chromatin remodelling machines, as well as of general transcription factors in this well characterized experimental setting.

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