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NF-Y binds to both G1- and G2-specific cyclin promoters; a possible role in linking CDK2/Cyclin A to CDK1/Cyclin B

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We previously reported that CDK2/Cyclin A can phosphorylate and activate the transcription factor NF-Y. In this study, we investigated a potential regulatory role for NF-Y in the transcription of *Cyclin A* and other cell cycle regulatory genes. Gel-shift assays demonstrate that NF-Y binds to CCAAT sequences in the *Cyclin A* promoter, as well as to those in the promoters of cell cycle G2 regulators such as *CDC2*, *Cyclin B* and *CDC25C*. Furthermore, expression of Cyclin A increases NF-Y's affinity for CCAAT sequences in the *CDC2* promoter; however, Cyclin A's induction of *CDC2* transcription is antagonized by p21, an inhibitor of CDK2/Cyclin A. These results suggest a model wherein NF-Y binds to and activates transcription from the *Cyclin A* promoter, increasing cellular levels of Cyclin A/CDK2 and potentiating NF-Y's capacity for transcriptional transactivation, and imply a positive feedback loop between NF-Y and *Cyclin A*/CDK2. Our findings are additionally indicative of a role for Cyclin A in activating Cyclin B/CDK1 through promoting NF-Y dependent transcription of *Cyclin B* and *CDC2*; NF-Y mediated crosstalk may therefore help to orchestrate cell-cycle progression. [BMB reports 2011; 44(8): 553-557]

INTRODUCTION

Orderly progression of the cell cycle is effected through the periodic activation and inactivation of a series of cyclin-dependent kinases (CDKs). CDK activities can be regulated through phasic accumulation and degradation of their cognate cyclins, their transient nuclear localization and/or phosphorylation, and their association with different CDK inhibitors (CKIs) (1, 2). In turn, cyclin/CDK complexes maintain cell-cycle control by phosphorylating key regulatory proteins with precise timing. Identification of cyclin/CDK substrates in specific phases of the cell cycle is necessary to a full understanding of cell growth and division.

It is well established that retinoblastoma (Rb) tumor suppressor proteins coordinate the sequential activation of CDK4 and CDK2 during the G1/S transition (3, 4). The CDK4/Cyclin D complex phosphorylates Rb, promoting Cyclin E expression through its E2F-dependent transcriptional activation and disruption of the Rb-HDAC complex, culminating in CDK2 kinase activation as S phase progresses (5, 6). In contrast, the factors involved in the handoff from CDK2 to CDK1 remain unidentified. However, previous work by our group and others demonstrates that overexpression of p53 blocks the G2/M transition (7, 8) and inhibits cell cycle-dependent transcription of *CDC2* and *CCNB* genes with consequent CDK1 inactivation (7). Roles for p21 and CDK2 at G2/M have been also suggested (9, 10), and Guadagno and Newport (10) have reported that inhibition of CDK2 by p21 results in a mitosis block and CDK1 inhibition in *Xenopus* oocyte extracts.

The NF-Y transcription factor (also known as CBF or CP1) binds the Y box of MHC class II Ea promoter (11). Its three subunits, YA, YB, and YC, are collectively required for NF-Y DNA-binding activity (12); NF-Y has been detected in almost all cell types, which was initially construed to mean that it exemplified an immutable, constitutive transcription factor; however, more recently it has been demonstrated that NF-Y's affinity for DNA alters over B-cell lifespan, potentially offering a means to fine-tune the subset of its target genes expressed during development (13) senescence (14, 15), and differentiation (16-18).

NF-Y is also required for transcriptional transactivation at G2/M. We previously reported that its YA subunit is the physiological substrate of the CDK2-Cyclin A complex (19, 20). YA phosphorylation appears essential to NF-Y's DNA-binding activity, and its peak phosphorylation during the cell cycle overlaps CDK2's activation window (20). Phosphorylation-deficient YA mutants (YA-aa) prevent transcription of NF-Y targets, including G2-specific genes such as *CDC2, CDC25C,* and *CCNB*, resulting in cell-cycle arrest at both G1 and G2/M (19). As YA-aa expression abrogates CDK1 production, CDK2- dependent YA phosphorylation appears to be essential for timely *CDC2* transcription (19). Based on these results, we propose a novel CDK network, in which CDK2, by phosphorylating NF-Y, enables *CDC2* transcription and hence CDK1 gene product activation. Here we demonstrate NF-Y's potential as a

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molecular linker for sequential activation of CDK2 and CDK1.

RESULTS

NF-Y is known to associate with and positively regulate transcription at CCAAT motifs in the promoters of cell cycle-regulatory genes. In this study, we performed electrophoretic mobility gel shift assays (EMSAs) to query NF-Y recruitment to the distal CCAAT motifs of the *CDC2* promoter. A 24 bp oligonucleotide was incubated with HepG2 hepatocarcinoma-cell nuclear lysate and analyzed by non-denaturing polyacrylamide gel electrophoresis (Fig. 1). Incubation with a random competitor oligonucleotide (poly [dI-dC]) evinced two major, high molecular-weight, bands (Fig. 1). The intensities of both upper and lower bands were diminished by the addition of unlabeled DNA fragments containing dCAT, the distal CCAAT sequence of *CDC2* promoter, but not by those containing dCAT mutant sequence (dCATm) or proximal CCAAT (pCAT) sequences of *CDC2* promoter, implying that the bands represent proteins that bind specifically to the distal CCAAT motif of the *CDC2* promoter (Fig. 1). The intensities of these bands were also markedly reduced by competition with unlabeled DNA oligonucleotides containing the distal and middle CCAAT sequences of the *Cyclin B* and *CDC25C* promoters, respectively, but not those with proximal CCAAT sequence of *Cyclin B* promoter. Interestingly, only the intensity of the upper band was lessened by challenge with cold DNA probes containing distal and proximal CCAAT sequences of *CDC25C* promoter or the single CCAAT sequence found in the *Cyclin A* promoter. This upper band therefore appeared to include a protein or proteins with affinity to CCAAT sequences in a range of cell-cycle specific promoters, in confirmation of our previous observation that HepG2 lysate contains a high-molecular weight protein or complex that can bind the *CDC2* promoter's distal CCAAT motif (20, 21).

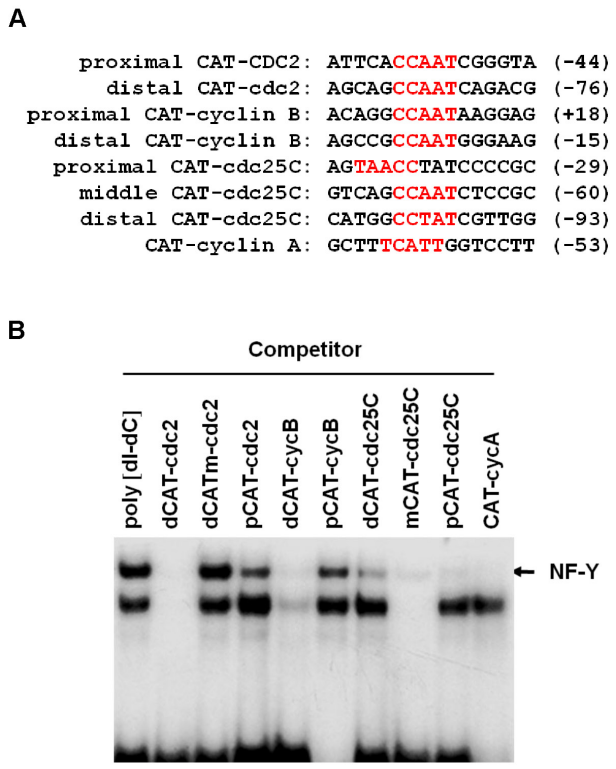

Fig. 1. Transcription-factor binding to CCAAT motifs in promoters of cell cycle-regulatory genes. EMSAs of complexes formed with DNA probes containing the *CDC2* promoter's distal CCAAT (sequences between -87 and -67 nt from the transcriptional start site). An end-labeled DNA probe was incubated with nuclear lysates from HepG2 cells. For competition assays, 100- or 500-fold molar excess of competitor oligonucleotides containing CCAAT sequences from the promoters of *CDC2, CCNB, CDC25C,* and *CCNA* genes was added to reactions. Where promoters exhibited multiple CCAAT sequences (*CDC2* and *CDC25C*), transcription-factor binding sites were named proximal, middle, distal, or mutated distal CCAAT element (dCAT, mCAT, pCAT, and dCATm, respectively, indicated above each lane); each was separately incubated with labeled DNA probe.

To assess the possibility that we were detecting NF-Y association with CCAAT boxes, we performed a gel shift assay with antibodies that recognize the transcription factor's YA subunit (Fig. 2). As in Fig. 1, we observed the appearance of two principal high molecular weight bands in reactions incubated with a probe containing distal *CDC2*-promoter CCAAT sequence, but only the upper band (band *a*) in incubations with CCAAT sequences from *Cyclin B*, *CDC25C*, and *Cyclin A*-promoters (Fig. 2); addition of antibodies against YA led to the formation of a supershifted band (band *b*) and the disappearance of band *a* for all tested CCAAT sequences (Fig. 2). Thus, these results indicate that band *a* contains NF-Y, and that NF-Y possesses the capacity to bind CCAAT motifs in the promoters of the cell-cycle regulators.

Since our previous findings had demonstrated that CDK2-Cyclin A could phosphorylate and activate NF-Y, we next examined whether expression of G1 cyclins such as A, D, or E could enhance NF-Y binding to the *CDC2* promoter's distal CCAAT sequence. EMSAs showed that NF-Y recruitment to

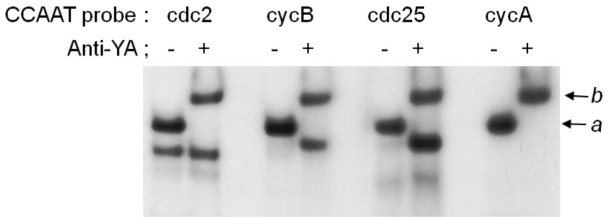

Fig. 2. NF-Y binding to *CDC2, CDC25C, CCNA,* and *CCNB* promoters. An antibody (1 μg) against YA was pre-incubated with nuclear lysate. The arrows indicate the complex formed at the distal CCAAT site (*a*) and a supershifted band formed in the presence of antibody (*b*).

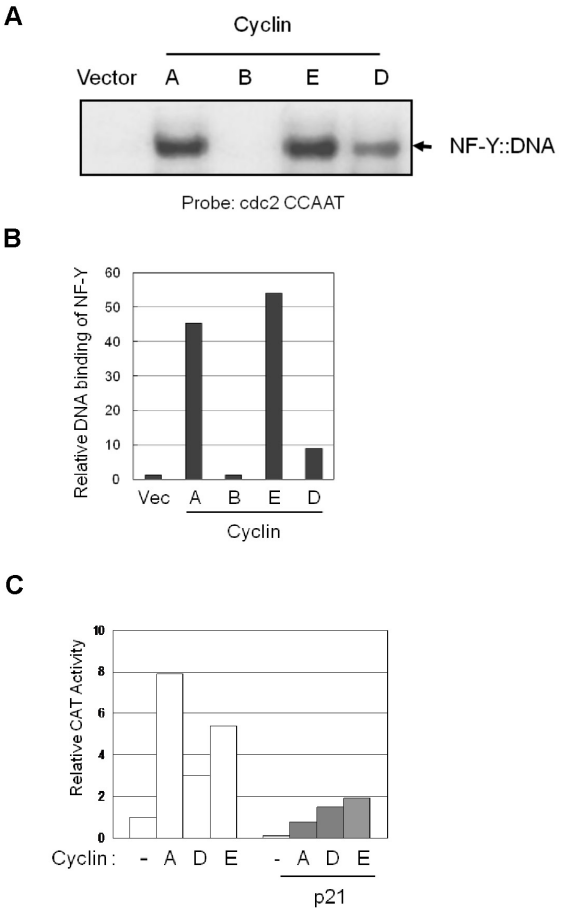

Fig. 3. G1 cyclins activate NF-Y DNA-binding activity. (A) HepG2 cells were transiently transfected with DNA encoding Cyclin A, D, or E, and nuclear lysates were prepared for EMSA using CCAAT motifs from the *CDC2* promoter. (B) Relative DNA-binding activity of NF-Y as in (A). The DNA binding activity of NF-Y in cells transfected with empty vector was adjusted to 1. (C) CAT activity in cells harboring empty vector was adjusted to 1. Relative CAT activity was determined as described in Experimental Procedures. The data represent averaged results from two independent transfections.

this CCAAT motif was significantly improved by expression of Cyclins A and E, but to a lesser extent by that of Cyclin D (Fig. 3A, B). Furthermore, Cyclins A and E could also augment NF-Y mediated transcription from the *CDC2* promoter, although Cyclin D did not (Fig. 3C). However, this effect on *CDC2* transcription could be suppressed by expression of p21, a CDK2 inhibitor (Fig. 3C). These findings are consistent with a model wherein Cyclin A/CDK2 can activate CDC2/Cyclin B through NF-Y phosphorylation, which induces the transcription of *CDC2*, *Cyclin B* and *CDC25C*.

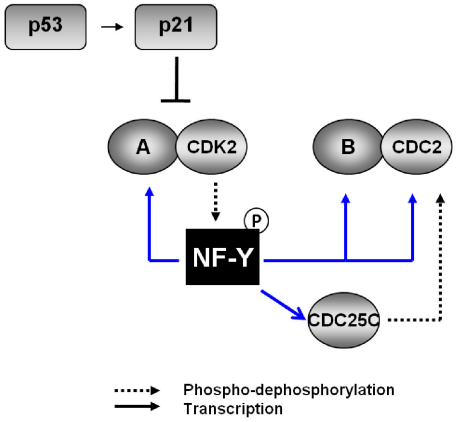

Fig. 4. Schematic representation of a positive feedback loop between NF-Y and CDK2 activation and a role for NF-Y as a molecular linker between CDK2 and CDK1. NF-Y binds to and activates the promoters of *CCNA* and G2-specific genes such as *CDC25C, CDC2,* and *CCNB*. Because Cyclin A/CDK2 phosphorylates and activates NF-Y, a positive feedback loop is established between Cyclin A/CDK2 and NF-Y, which additionally participates in sequential activation of CDK2 and CDK1.

DISCUSSION

We previously reported that YA-aa, which dominant-negatively inhibits NF-Y's transcriptional transactivation abilities, inactivates CDK2 as well as CDC2, causing cell-cycle arrest at the G2/M transition (19). In the present study, we show that Cyclin A increases NF-Y's affinity for CCAAT sequences in the *CDC2* promoter, which suggests that NF-Y mediated transcription of *Cyclin A* can positively regulate the cellular levels of both CDK2/Cyclin A and CDC2/Cyclin B. YA phosphorylation and CDK2 activation therefore appear to create a positive-feedback loop (Fig. 4).

We also demonstrate that Cyclin A promotes NF-Y binding to CCAAT sequences in the *CDC2* promoter, concomitant with *CDC2* transcription (Fig. 3). Our findings therefore support activation of Cyclin B/ CDC2 through Cyclin A mediated potentiation of NF-Y transcription at G2-specific promoters, including those for *CDC2*, *Cyclin B* and *CDC25C*.

MATERIALS AND METHODS

Cell culture, transfection, and CAT assay

The EJ-p53 cell line, in which tetracycline regulates p53 expression, has been previously described (22). HepG2 cells were grown in 10% FBS/Dulbecco's modified Eagle's medium (DMEM, VENDOR, CITY, COUNTRY) according to published protocols (23). DNA transfection was performed using the $CaPO_4$ coprecipitation procedure (24). Cells were harvested and proteins extracted by three cycles of freeze-thawing at 48 hr post-transfection. Lysate protein concentrations were determined with the Bio-Rad protein assay kit (Bio-Rad, CITY,

COUNTRY). In all transfection experiments, β-galactosidase activity, derived from transcription of pCMV-β-gal or pMT-β-gal, was used both to monitor and to normalize transfection efficiency. CAT and β-galactosidase assays were carried out according to the protocol of Gorman *et al*. (25). The promoter activities of reporter constructs were assayed by measuring radioactivity of acetylated forms that were counted directly on a PhosphorImage Analyzer, BAS-1500 (Fuji, CITY, COUNTRY).

Electrophoretic mobility shift assay

Nuclear lysates were prepared according to the method of Dignam *et al*. (26). A double-stranded, *in vitro* synthesized DNA fragment containing the distal CCAAT motif (-83 to -75) was labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Nuclear lysates (5 μg) were pre-incubated for 30 min at 0°C [or on ice] with 1 μg of poly [dI-dC] (Pharmacia Biochemical Inc., CITY, COUNTRY) and unlabelled competitor DNA in buffer containing 25 mM HEPES (pH 7.9), 20 mM KCl, 30 mM NaCl, 0.5 mM EDTA, 0.25 mM DTT, and 10% glycerol; after end-labelled probe (about 15,000 cpm) was added, incubation continued for an additional 20 min at room temperature. DNA-protein complexes were separated on a 6% polyacrylamide gel in 0.25X TBE at 15 mA for 2 hrs. For antibody supershift assays, 1 μg of each antibody specific to the YA subunit of NF-Y (KB070 and KB090, Accurate, CITY, COUNTRY) was included in the pre-incubation mixture. The DNA sequence of in vitro synthesized oligomers used as competitors was 5'-CTGGGCTCTGATTGGCTGCTTTGAA-3' for distal CCAATT.

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