Transactivation of the Human cdc2 Promoter by Adenovirus E1A

E1A INDUCES THE EXPRESSION AND ASSEMBLY OF A HETEROmeric COMPLEX CONSISTING OF THE CCAAT BOX BINDING FACTOR, CBF/NF-Y, AND A 110-kDa DNA-BINDING PROTEIN*

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Cyclin-dependent kinases (CDKs) play an important role in the eukaryotic cell cycle progression. Cdc2 (CDK1) is expressed in late G1/S phase and required for G2 to M phase transition in higher eukaryotes. The oncoproteins, SV40 large T antigen and adenovirus E1A, induce a 110-kDa protein which specifically recognizes the two inverted CCAAT motifs of the cdc2 promoter in cycling cells and plays an essential role in transactivation of the human cdc2 promoter. Since these CCAAT motifs also conform to the consensus binding sites for the ubiquitous heterotrimeric transcription factor, CBF/NF-Y, the role of CBF/NF-Y in the transactivation of the cdc2 promoter was examined in this study. Our results indicate that CBF/NF-Y and the 110-kDa protein interact with the CCAAT box motif to form a heteromeric complex. However, mutagenesis of the pentanucleotide CCAAT motif or in the presence of urea greater than 2.5 M, no heteromeric complex was formed. In contrast, the 110-kDa protein could still bind the mutant CCAAT motif or with the wild type motif in the presence of 2.5 M urea. Furthermore, E1A.12S induced the gene expression of all three subunits of CBF/NF-Y. Coexpression of E1A and a dominant negative mutant NF-YA subunit significantly reduced the E1A-mediated transactivation of the cdc2 promoter in a dose-dependent manner. These results support the conclusion that E1A protein mediates optimal transactivation of the human cdc2 promoter by inducing the expression and assembly of a heteromeric complex consisting of the 110-kDa protein and the CBF/NF-Y which interacts with the two CCAAT motifs of the cdc2 promoter.

The progression of the eukaryotic cell cycle through quiescent to proliferating state is tightly controlled by a periodic expression and function of the key regulatory proteins, such as cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors. The p34CDC2 (CDK1), the product of cdc2 gene in S. pombe or the cdc28 gene in Saccharomyces cerevisiae, is the first identified CDK. This CDK1 regulates both the G1 → S and G2 → M phase transitions (1–9) (for reviews, see Refs. 10–13, and the references therein). In higher eukaryotes, separate CDKs are involved in these cell cycle transitions; the homologue of CDK1 is expressed in G1 → S phase and is primarily required for the G2 → M phase transition (7–9). Other CDKs are involved in G1 → S phase transitions (8, 14, 15) although the CDK1 also seems to play a role in phosphorylation of key substrates involved in transition of the G1 → S phase (for reviews, see Refs. 12 and 16, and references therein).

The kinase activity of CDK1 is regulated by phosphorylation and dephosphorylation, and is linked to the growth state of the cells (4) (for reviews, see Refs. 12, 13, and 16). Moreover, the expression of human cdc2 mRNA and the CDK1 levels are very low in cells which are under growth arrest, differentiation, or development. When the cells enter the cell cycle, the cdc2 gene expression is increased dramatically (17–22) suggesting that the CDK1 is required for proliferation. The low levels of the cdc2 gene expression in growth arrested baby rat kidney cells are also dramatically reversed by expression of the oncoproteins of DNA tumor viruses, adenovirus E1A (E1A) and SV40 large T antigen (SV40-LT). This activation of the cdc2 gene expression is accompanied by induction of p34CDC2 kinase activity, pRB phosphorylation, and DNA synthesis (22–24).

The growth promoting activities of the DNA tumor virus oncoproteins are at least in part attributable to their ability to associate with proteins that negatively regulate cell growth, the retinoblastoma susceptibility gene product (pRB) and p53 (25–28) (for reviews, see Refs. 29–31). For example, interaction of E1A and SV40-LT with pRB disrupts the transcriptionally inactive pRB-E2F complexes resulting in transcriptional activation of growth promoting E2F-dependent genes (26, 32–35) (reviewed in Refs. 29–31). An independent pathway by which E1A promotes cell growth is by its association with p300/CREB binding protein which is required as a coactivator of p53 and inhibition of p53-dependent p21WAF1/CIP1 expression resulting in the release of cells from G1 arrest and in stimulation of cellular DNA synthesis (36–40). The association of E1A with p21WAF1 also has been shown to overcome the transforming growth factor-β1-mediated growth arrest at G1 phase of the cell cycle (41).

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1 The abbreviations used are: CDK, cyclin-dependent kinase; c/EBP, CCAAT/enhancer-binding protein; E1A, rat liver epithelial cell; E1A, electrophoretic mobility shift assay; h, base pairs; PAG, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase; CDE, cell cycle-dependent element; CHR, cell cycle homology region.
Previous studies in our laboratory showed that E1A and SV40-LT transactivate the human cdc2 promoter in cycling cells by inducing a 110-kDa protein which specifically recognizes the two-inverted CCAAT box (ATTGG in the opposite strand) motifs in the cdc2 promoter located at positions −74 to −78 and −42 to −46 with respect to transcription start site (42, 43).

The CCAAT motifs are present in many eukaryotic class II promoters and are also specific binding sites for the ubiquitously expressed heterotrimeric transcription factor, NF-Y (CBF/NF-Y). CBF/NF-Y consists of three subunits (A, B, and C) which are all required for DNA binding activity (44–47). Moreover, NF-Y is known to function in most cases in conjunction with a variety of specific transcriptional activators to achieve optimal levels of gene expression. For example, in albumin promoter, NF-Y and CCAAT/enhancer-binding protein (C/EBP) are both required for optimal level of liver-specific gene expression (48). The transcriptional regulation of the 3-hydroxy-3-methylglutaryl-CoA synthase gene involved in cholesterol bio-synthetic pathway is achieved by the sterol regulatory element-binding proteins together with CBF/NF-Y through sterol regulatory element and the CCAAT box motif in the promoters of target genes. A direct interaction between sterol regulatory element-binding proteins and CBF/NF-Y was demonstrated in that study (49).

In this study, we show that CBF/NF-Y interacts with the 110-kDa protein induced by adenovirus E1A.12S. The formation of this heteromeric complex is disrupted by mutagenesis of the pentanucleotide CCAAT motif or by treatment with urea greater than 2.5 M. The 110-kDa protein, however, could still bind the mutant DNA fragment or the wild type DNA fragment in the presence of 2.5 M urea indicating that the protein binds to the flanking sequences of the pentanucleotide CCAAT motif. Hence, it is referred as the CCAAT box-associated factor in this study (CBAF/cdc2). Furthermore, we show that expression of E1A increases the steady state levels of mRNAs encoding all three subunits of CBF/NF-Y. In addition, we present evidence that the heterotrimeric CBF/NF-Y plays an important role in transactivation of the human cdc2 promoter by E1A based on the results that the expression of a dominant negative mutant CBF/NF-Y interferes with this transactivation in a dose-dependent manner. Our results, taken together, suggest that E1A mediates optimal transactivation of the human cdc2 promoter by inducing both the heterotrimeric CBF/NF-Y and the 110-kDa CBAF/cdc2 as the costimulatory factor for NF-Y.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Human embryonic kidney cells transformed by early region 1 of adenovirus (293 cells) were grown in Joklik's modified Eagle's medium supplemented with 10% heat-inactivated horse serum (Life Technologies, Inc.) in spinner suspension flasks. Fisher rat liver epithelial cell line (RLE) cells were grown in Dulbecco's modified Eagle's medium/F12 medium (Sigma) supplemented with 10% fetal bovine serum and 50 μg/ml each of streptomycin and penicillin. Cells were harvested at 37 °C in humidified air containing 5% CO2.

**Preparation of Nuclear Extracts—**For preparation of nuclear extracts, RLE cells in 80% confluence were infected with adenovirus encoding E1A.12S (22); kindly provided by Dr. Elizabeth Moran, Temple University) at 50 plaque forming units/cell. Cells were harvested 48 h post-infection and nuclear extracts were prepared as described previously (42).

**Electrophoretic Mobility Shift Assay—**Electrophoretic mobility shift assays (EMSA) were carried out as described previously (42). The double-stranded DNA was labeled at the 5′-end by T4 polynucleotide kinase and γ32PATP following standard procedures (50). The oligonucleotides used in this study are: 1) the distal CCAAT element of the human cdc2 promoter (5′-CATGGGCTCTGATTGGCTTTG-3′), 2) (Mut-CCAAT) probe containing mutated CCAAT box motif (5′-CATTGAGGCCTGGCTTTG-3′), 3) probe containing the proximal CCAAT/box-binding motif of the human cdc2 promoter (5′-GCTACCGATTGTGATCCGG-3′). As a control for competition experiments in gel mobility assays, the unrelated oligonucleotide containing the E2F-like binding site of p21WAF1 promoter (5′-CAAGCTGCCTCCGCGTGGCCAGCAGC-3′) was used. For EMSA, nuclear extracts (5 μg/lane) were mixed in 20 μl of reaction buffer (25 mM Heps-KOH, pH 7.9, 5 mM KCl, 150 mM EDTA, 0.15% gelatin, 2% CHAPS, 5 μg/ml dithiothreitol, 10% glycerol, 0.1 mg/ml poly(dI-dC) with the labeled probe (5 × 106 cpm/lane). The reaction mixture was incubated at 37 °C for 30 min, and stopped by adding 2 μl of stop solution containing 50 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, and 5% glycerol. The DNA-protein complexes were fractionated by electrophoresis on 4% to 7% polyacrylamide gels. The gels were dried and subjected to autoradiography using x-ray films (NEN Life Science Products Inc.).

For supershift experiments, EMSA reaction mixtures were incubated with 1.5 to 5.0 μg of rabbit anti-NF-YA or NF-YB antibody (Rockland, Gilbertsville, PA) for 1 h on ice prior to addition of the probes.

**UV Cross-linking of DNA-Protein Complexes—**UV cross-linking was carried out (61) as follows. A 29-bp DNA probe was made using Klenow DNA polymerase-catalyzed fill-in reaction in the presence of 5-bromo-2′-deoxyuridine triphosphate (Sigma) and [α-32P]dCTP (NEN Life Science Products) and dNTPs. The two primers used were partially complementary oligonucleotides with the sequences of 5′-CATGGGCTCTGATTGGCTTTG-3′ and 5′-GGGGCGAAAGC-GA-3′. The labeled probe was added to the EMSA reaction mixture containing 20 μg of the nuclear extracts and 200 μg of DNA-protein complexes were fractionated by 4% nondenaturing gel. The DNA-protein complexes were located by autoradiography by exposing the gel to the x-ray film overnight at 4 °C. The band was excised and exposed to 285 nm UV light for 15 min at 4 °C. Then the excised gel fragment was mixed with 2 × SDS-PAGE loading buffer and blotted in the well of a 4% stacking polyacrylamide gel polymerized over a 6% resolving gel containing 0.1% SDS. After electrophoresis, the UV cross-linked DNA-protein complexes were detected by autoradiography.

**Southern Blot Analysis—**The conditions used for Southern blot analysis were as described previously (42). Briefly, 10 μg of nuclear proteins from 293 cells were loaded in each lane and were fractionated by 8.0% SDS-PAGE. The proteins were transferred electrophoretically to a polyvinylidine difluoride membrane (Bio-Rad). After denaturation and renaturation steps, the proteins immobilized on the membrane were incubated with the 5′-[32P]-labeled distal or proximal cdc2/CCAAT probe (106 cpm/ml). The membranes were washed and subjected to autoradiography.

**Immunoprecipitation—**The nuclear extracts from 293 cells were precleared by incubation with normal rabbit serum followed by treatment with goat anti-Agaroase beads (Life Technologies, Inc.). The precipitant obtained after centrifugation was incubated with anti-NF-YA antibody, normal rabbit serum, or an unrelated antiserum raised against hepatitis C virus NS5A protein. The precipitated protein complex was recovered by protein A-agarose beads. The immunoprecipitated complexes were subsequently subjected to Southern blot analysis using 5′-[32P]-labeled distal or proximal cdc2/CCAAT probe to detect the 110-kDa CBAF/cdc2.

**Northern Blot Analysis—**Total RNAs were obtained from the adenovirus-infected RLE cells 32 h post-infection by using Trizol (Life Technologies, Inc.). Twenty micrograms of the RNAs were fractionated on 1% agarose, 6% formaldehyde gel and transferred onto nitrocellulose membrane. After heating the membrane at 80 °C for 2 h, the membrane was hybridized with a 32P-labeled probe using the Quick-Hyb hybridization solution. Probes used for the Northern blottings were rat NF-Ya (CBF-B) and NF-Yb (CBF-A) and NF-Yc cDNAs (pCITE2a-CBF-A, -B, and -C) were kindly provided by Dr. S. Maity, University of Texas, M.D. Anderson Cancer Center). For detection of the cdc2 mRNA the probe used was a DNA fragment derived from the cDNA encoding human cdc22 by digesting the pCMV1-cdc2 plasmid (kindly provided by Dr. J. Campisi, Lawrence Berkeley Laboratory, University of California) with BglII and KpnI.

**Chloramphenicol Acetyltransferase Assay (CAT)—**RLE cells (in 45% confluence) in 25-cm2 culture flasks were infected with recombinant adenovirus expressing wild type 125.E.1A.1A. One hour after infection, cells were transfected with cdc2 promoter-CAT reporter plasmids (cdc2-CAT) (22 μg/ml, 42) and nuclear extracts from the cells in 150 μg/ml nuclear extracts. The results of dominant negative analysis of NF-YA (NF-YA29) (51) for 6 h in serum-free medium in the presence of cationic lipids as described previously (42). The NF-YA29 dominant negative expression plasmid was generously provided by Dr. Timothy F. Osborne, University of California-Davis, with kind permission from Dr. Robert Mantovani from the University of De Giusti Studi Di Milano, Italy. Cells were incubated for 40 h in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and cell lysates

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were prepared for CAT assays as described previously (42). E. coli β-galactosidase expression plasmid was co-transfected as the internal control to monitor and normalize the transfection efficiencies.

RESULTS

**Human cdc2 Promoter Contains Two Inverted CCAAT Box Motifs Which Conform to Consensus NF-Y-binding Sites and Are Recognized by the 110-kDa CBAF/cdc2 and NF-Y**—The two inverted CCAAT box motifs of the human cdc2 promoter conform to the consensus NF-Y-binding site, TGATTG(T/C)-(T/C)A/G, derived from a random polymerase chain reaction-based selection procedure (52). Our previous studies indicated that a 110-kDa protein (referred to as CBAF/cdc2 in this study) was induced by the oncoproteins, SV40-LT and E1A, and it was overproduced in cells transformed by SV40 and adenovirus E1 region (COS and human 293 cells, respectively). This protein specifically bound the 180-bp DNA fragment containing both CCAAT motifs or a 24-bp fragment containing the distal motif (42, 43). Therefore, we sought to determine whether DNA-protein complexes formed between the distal CCAAT motif of the cdc2 promoter and the nuclear extracts of human 293 cells also contained the ubiquitous CBF/NF-Y. As shown in Fig. 1A (lane 2), the nuclear extracts from human 293 cells formed three major complexes (C-I, -II and -III) with the 32P-labeled distal CCAAT motif used as a probe in EMSA. The specificity of these DNA-protein complexes formed with the CCAAT motif was established by competition experiments using unlabeled oligonucleotides containing wild type CCAAT, unrelated E2F-binding site, or the mutated CCAAT motif (lanes 3–5, respectively). To determine whether any of these DNA-protein complexes also contained CBF/NF-Y, supershift assays in which rabbit polyclonal antibodies raised against CBF/NF-YA and CBF/NF-YB subunits were incubated with the DNA-protein complexes. The results shown in Fig. 1B (lanes 3 and 5) indicated that the antibodies against NF-YA and NF-YB supershifted the complex I, although the commercial anti-NF-YB was much less efficient than the anti-NF-YA. However, both antibodies had no effect on complexes II and III, which suggested that these complexes do not include NF-Y. Moreover, incubation with normal rabbit serum used as control (Fig. 1B, lanes 2 and 4) had no effect. These results indicated that the DNA-protein complex I contains CBF/NF-Y. We did not test for the presence of CBF/NF-YC in this complex because a suitable antibody against the C subunit was not available for this study. However, the complex I is likely to include the CBF/NF-YC as previous studies have shown that all three subunits are required for DNA binding (47).

Next we analyzed whether the complex I also included the 110-kDa CBAF/cdc2 protein using the UV cross-linking method. First, 32P- and 5-bromodeoxycytidine-labeled cdc2 CCAAT motif was used for EMSA. Subsequent to EMSA, autoradiographic detection of the three major DNA-protein complexes (C-I to C-III), the complex I band was cut out from the polyacrylamide gel. The gel piece was exposed to UV as the internal control to monitor and normalize the transfection efficiencies.

[FIG. 1. Interaction of the 110-kDa CBAF/cdc2 and NF-Y. A, EMSA using nuclear extracts from human 293 cells were carried out with the labeled oligonucleotide containing the distal wild type CCAAT box motif of the human cdc2 promoter (dis WT CCAAT) as described under “Experimental Procedures.” The positions of complex I, II, and III were indicated (lane 2). Lane 1 contained the free probe in the absence of nuclear extracts. The competition experiments (comp.) were carried out by adding a 100-fold excess of unlabeled cold oligonucleotide containing the distal wild type CCAAT box motif of the human cdc2 promoter (WT cold), mutated CCAAT box motif (Mut cold), and E2F-binding site motif (E2F cold), and mutated CCAAT box motif (Mut cold) in lanes 3–5, respectively. B, supershift assay was carried out by incubation of nuclear extracts with antibodies against NF-YA and NF-YB (lanes 3 and 5, respectively), 60 min before adding the labeled distal wild type CCAAT motif as the probe. Normal rabbit serum was used as the control (lanes 2 and 4). C, UV cross-linking using nuclear extracts from human 293 cells were carried out as described under “Experimental Procedures” using the labeled distal wild type CCAAT motif as the probe. In lane 4, two major bands, 110 and 45 kDa, were observed in UV cross-linking of complex I (C-I) isolated from an EMSA. Lane 3 represents the C-I without UV cross-linking. Lanes 1 and 2 represent the free probe without and with UV irradiation. D, first, immunoprecipitations using 293 cell nuclear extracts were carried out using either normal rabbit serum (lane 1), anti-NF-YA antibody (lane 2), or anti-hepatitis C virus NSSA antibody as an unrelated antibody control. The immune complexes were subjected to Southwestern blot analysis using the labeled distal wild type CCAAT as the probe as described under “Experimental Procedures.” In lane 2, the 110-kDa CBAF/cdc2 was detected in the immune complex with anti-NF-YA antibody.]

PAGE and Southwestern blot analysis using 32P-labeled distal CCAAT motif of the cdc2 promoter as the probe. As shown in Fig. 1D (lane 2), the results from Southwestern blot analysis showed that only the anti-NF-YA immune complex from 293 cells contains the 110-kDa CBAF/cdc2 protein (lane 2), but not the immune complexes obtained using normal rabbit serum or an unrelated antisera raised against hepatitis C virus NSSA protein (lanes 1 and 3, respectively). The results taken together indicated that the 110-kDa CBAF/cdc2 and the CBF/NF-Y formed a heteromeric complex with the DNA frag-
ment containing the distal CCAAT motif of the cdc2 promoter.

Since the 293 cells are constitutively expressing proteins encoded by the adenovirus E1 region, which encodes E1A and E1B proteins (53), we sought to determine whether this heteromeric complex I is induced by E1A alone. For these experiments, RLE cells were used for expression of E1A protein by infection with a recombinant adenovirus encoding E1A.12S. The nuclear extracts prepared from the infected RLE cells were analyzed by EMSA. As shown in Fig. 2, the endogenous levels of the 110-kDa CBAF/cdc2 (Fig. 2B, lane 1) as well as the formation of complex I in EMSA (Fig. 2A, lane 2) were barely detectable in the absence of E1A expression in RLE cells. The results shown in Fig. 2A indicate that the formation of the heteromeric DNA-protein complex I was also induced by transient expression of E1A.12S protein in RLE cells (lane 3). Evidence that this complex I also contained CBF/NF-Y was obtained by supershift assays performed by incubation with antibodies against NF-YA and NF-YB (lanes 5, 6, and 8) whereas incubation with normal rabbit serum had no effect (lane 4). Furthermore, it is shown that E1A induces the 110-kDa CBAF/cdc2 in RLE cells by the Southwestern blot analysis (Fig. 2B, lane 2).

Adenovirus E1A Induces Gene Expression of All Three Subunits of CBF/NF-Y—These results indicated that the DNA-protein complexes containing the heterotrimeric NF-Y protein and the 110-kDa CBAF/cdc2, which interact with each other and with the CCAAT box motif of the cdc2 promoter, were induced by the expression of adenovirus E1A protein. Next we sought to determine whether the induction of the cdc2 gene expression by E1A is concomitant with the induction of the heterotrimeric CBF/NF-Y gene expression. Total RNAs isolated from RLE cells infected with recombinant adenovirus encoding E1A.12S protein were used for Northern blot analysis. The results shown in Fig. 3A indicate that the steady state levels of cdc2 mRNA were increased after the expression of E1A in RLE cells as expected based on previously reported observations in quiescent baby rat kidney cells (18, 22, 23) and in cycling RLE cells (43). In addition, we observed that the steady state levels of mRNAs encoding all three subunits of CBF/NF-Y were increased in the E1A expressing cells (Fig. 3B).

CBF/NF-Y and the 110-kDa Protein Recognize Distinct Sequences in the Human cdc2 Promoter—The results of our immunoprecipitation with anti-NF-YA antibody followed by Southwestern blot analyses (Fig. 1C) and EMSA (Fig. 1A) provided evidence that the 29-bp distal CCAAT motif of the cdc2 promoter recognized the 110-kDa protein and that it interacts with the heterotrimeric CBF/NF-Y. These observations raised a question regarding the sequences recognized by both proteins. Previous studies on the DNA sequences recognized by CBF/NF-Y indicated that all three subunits of CBF/NF-Y and the pentanucleotide CCAAT motif are stringently required for DNA binding (47, 54). In addition, the flanking sequences of the pentanucleotide motif have been shown to stabilize NF-Y binding (52, 54).

To address this question, we carried out EMSA using a mutant 24-bp DNA fragment as the labeled probe which contained mutations in the CCAAT motif but retained the flanking sequences as wild type (Mut-CCAAT). The results shown in Fig. 4A indicated that the heteromeric complex I was not formed with the Mut-CCAAT motif (Fig. 4A, lane 4). However, the DNA-protein complex formed with the Mut-CCAAT fragment had a faster electrophoretic mobility than that of complex I (as shown by an arrow in Fig. 4A). This fast migrating band could be competed out by unlabeled oligonucleotides such as the Mut-CCAAT (lane 5) and distal wild type CCAAT motif (lane 7), but not by an unrelated oligonucleotide such as the E2F-like binding site of the p21Cip1 promoter (lane 6; see also Fig. 6A, lane 4). We observed that the new band which appeared in the cold competition EMSA using the wild type CCAAT oligonucleotide (see Fig. 4A, lane 4) had similar mobility as the faster migrating band obtained with the Mut-CCAAT (Fig. 4A, lane 4). Therefore, we performed EMSA for a longer period and the results showed that these two bands were distinct DNA-protein complexes. Consistent with this observation, the new band observed in a cold competition experiment using the distal wild type CCAAT as the labeled probe (Fig. 1A, lane 3) could not be competed out by adding the Mut-CCAAT oligonucleotide (data not shown). The mutation of the pentanucleotide CCAAT motif in the Mut-CCAAT oligonucleotide would be expected to disrupt NF-Y binding based on the previous report (52). Therefore we sought to determine whether this
faster migrating DNA-protein complex contained the 110-kDa CBAF/cdc2. To this end, we used the 32P-labeled Mut-CCAAT fragment as a probe for the Southwestern blot analysis using nuclear extracts from 293 cells. The results of Southwestern blot analysis shown in Fig. 4B indicate that the Mut-CCAAT motif could still recognize the 110-kDa protein (lane 1). Moreover, binding of the 110-kDa protein to the Mut-CCAAT motif could be competed out by adding a 50-fold excess of cold Mut-CCAAT oligonucleotide (Fig. 4B, lane 2). These results, taken together, indicated that the 110-kDa CBAF/cdc2 interacted with the DNA sequences flanking the pentanucleotide CCAAT motif of the cdc2 promoter.

Our results indicated that the 110-kDa protein alone was capable of specifically binding the Mut-CCAAT distal motif of the human cdc2 promoter and that this DNA-protein complex had a faster mobility than that of complex I in EMSA (indicated by an arrow in Fig. 4A). Moreover, the faster migrating DNA-protein complex could also be detected in smaller amounts in EMSA carried out with the 293 cell nuclear extracts and the wild type DNA probe (Fig. 4A, lane 2). This observation suggested that there is some free 110-kDa CBAF in 293 cell extracts not part of the heteromeric complex with the CBF/NF-Y. Therefore, we sought to determine whether the heteromeric complex I which contains NF-Y and the 110-kDa CBAF (from the results of Southwestern blot analysis and UV cross-linking) could be dissociated to the faster migrating DNA-protein complex. To this end, we analyzed the stabilities of the DNA-protein complexes by EMSA in the presence of increasing concentrations of urea. The results shown in Fig. 5A indicated that urea concentrations exceeding 2.5 M significantly disrupted the interaction between NF-Y and the 110-kDa protein such that the complex I could no longer be formed. However, the faster migrating DNA-protein complex could still be formed in the presence of urea (shown by an arrow in Fig. 5A), and it had the same mobility as the DNA-protein complex formed with the Mut-CCAAT probe in the absence of urea (Fig. 4A, shown by arrow). The specificity of the DNA-protein complexes formed under conditions which contained urea in EMSA reaction mixtures was established by adding 100-fold excess of cold oligonucleotides containing either the wild type or the distal Mut-CCAAT motif. In Fig. 5B, the complex I, II, and III, and the faster migrating band were competed out by the cold wild type CCAAT oligonucleotide (lane 3); however, only the faster migrating band was competed out by the cold Mut-CCAAT oligonucleotide (lane 4).

The Proximal CCAAT Motif of the Human cdc2 Promoter Is Also Recognized by the Heteromeric Complex between NF-Y and the 110-kDa Protein—Since the proximal inverted CCAAT motif of the human cdc2 promoter has the consensus sequences for NF-Y binding, we analyzed the interaction of the heteromeric complex from 293 cell nuclear extracts with a 21-bp oligonucleotide probe containing the proximal CCAAT motif by EMSA. Interestingly, the results shown in Fig. 6A indicated that only complex I was formed with the proximal motif (lane 2) and this pattern was similar to the results obtained with the CCAAT element of the cyclin A promoter (see Ref. 62). This DNA-protein complex was competed out with cold wild type oligonucleotide (Mut-cold) or the Mut-CCAAT oligonucleotide (Mut-cold) were used for a competition experiment (Comp.). Complexes I, II, and III, and the faster migrating band were competed by the cold wild type oligonucleotide (lane 3); however, only the faster migrating band was competed out by the cold Mut-CCAAT oligonucleotide (lane 4).
complex formed with the proximal motif was confirmed by Southwestern blot analysis (Fig. 6B). This interaction is specific as shown by the result that a 50-fold excess of the unlabeled proximal CCAAT motif reduced this interaction significantly (Fig. 6B, lane 2).

NF-Y Plays an Important Role in Transactivation of the Human cdc2 Promoter by Adenovirus E1A Protein—Since our results showed that the oncoprotein E1A activates the gene expression of all three subunits of CBF/NF-Y, we sought to determine whether CBF/NF-Y plays any role in E1A-mediated transactivation of the human cdc2 promoter. Expression of E1A.12S results in transactivation of the human cdc2 promoter/CAT reporter construct in RLE cells (43). To test whether CBF/NF-Y is involved in E1A-mediated transactivation, RLE cells were infected with adenovirus wild type E1A.12S and then co-transfected with an expression plasmid encoding a dominant negative mutant NF-YA subunit (NF-YA29) (51). The details are described under "Experimental Procedures." The CAT assays were performed using the extracts from transfected cells. This experiment was repeated twice with similar results. The results of the CAT assays were quantitated by liquid scintillation counting of each sample and plotted in a bar graph. The degree of reduction by coexpression of NF-YA29 mutant was estimated by normalizing the CAT activity value expressed in the absence of the NF-Y mutant to 1.

FIG. 6. DNA binding activity of nuclear extracts from 293 cells to proximal wild type CCAAT box motif of cdc2 promoter. A, EMSA using nuclear extracts from human 293 cells were carried out with the labeled oligonucleotide containing the proximal wild type CCAAT box motif of the human cdc2 promoter as described under "Experimental Procedures." Only complex I was observed (lane 2). The competition experiments were carried out by adding a 100-fold excess of unlabeled cold oligonucleotide containing the wild type CCAAT box motif (prox-cold in lane 3) or the E2F-like binding site oligonucleotide (E2F-cold in lane 4). The complex I was competed out by the wild type proximal CCAAT motif but not by the E2F-cold oligonucleotide. Supershift EMSA assays were carried out by incubation of nuclear extracts with normal serum, anti-NF-YA, or anti-NF-YB antibody (lanes 5–7, respectively), 60 min before adding the labeled proximal wild type CCAAT oligonucleotide. B, Southwestern blot analysis using 293 cell nuclear extracts (20 μg/lane) was carried out using the proximal wild type CCAAT box motif as the probe as described under "Experimental Procedures." The 110-kDa CBF/cdc2 was detected similar to that obtained with the wild type distal CCAAT motif as the probe. The competition experiment was carried out by adding a 50-fold excess of unlabeled cold oligonucleotide.

FIG. 7. Functional assay for activation of cdc2 promoter by NF-Y. One hour after infection of recombinant adenovirus expressing 12S.E.1A, RLE cells were co-transfected with 3 μg of the cdc2 promoter/CAT reporter plasmid (cdc2-Taq1; see Ref. 42) with increasing amounts of dominant negative NF-YA29 mutant (51). The details are described under "Experimental Procedures." The CAT assays were performed using the extracts from transfected cells. This experiment was repeated twice with similar results. The results of the CAT assays were quantitated by liquid scintillation counting of each sample and plotted in a bar graph. The degree of reduction by coexpression of NF-YA29 mutant was estimated by normalizing the CAT activity value expressed in the absence of the NF-Y mutant to 1.

DISCUSSION

The transcriptional regulation of the human cdc2 promoter seems to be complex as it has multiple regulatory elements for binding of several transcription factors (18, 55). Within the −359 region of the cdc2 promoter, the ATF, c-Myb, Sp1, and E2F-binding sites as well as the two inverted CCAAT box motifs (at positions −74 to −78 and −42 to −46) contribute to the basal activity of the promoter (42). These CCAAT box motifs conform to the consensus binding sites for the heterotrimeric and ubiquitous transcription factor NF-Y (52) (for reviews, see Refs. 56 and 57).

Our previous studies indicated that the two CCAAT box binding motifs of the human cdc2 promoter were not only important for the basal activity of the promoter but also for the SV40-LT and adenovirus E1A-mediated transactivation of the promoter in cycling cells (42, 43). In those studies, a 110-kDa protein was shown to be induced by both viral oncoproteins. Using Southwestern blot analysis and EMSA, it was shown that this 110-kDa nuclear protein specifically bound the 180-bp DNA fragment spanning the two (both distal and proximal) CCAAT motifs of the human cdc2 promoter as well as the 29-bp NF-Y and the 110-kDa protein with the pentanucleotide CCAAT motifs and its flanking sequences.
DNA fragment containing only the distal CCAAT motif (42, 43). However, the mechanism of transcriptional activation of the cdc2 gene by these viral oncoproteins and the role of this 110-kDa protein or the CCAAT motifs of the cdc2 promoter in transcriptional activation remained unclear.

In this study we provide evidence that this 110-kDa protein is a component of a heteromeric complex with ubiquitous transcription factor, CBF/NF-Y. Furthermore, our evidence, obtained using a dominant negative mutant expression clone of NF-YA subunit (NF-YA29 (51)), indicates that CBF/NF-Y plays an important role in the regulation of E1A-mediated transcription of human cdc2 gene expression. This role of NF-Y in transcription of a cell cycle-regulated cdc2 gene by an oncprotein, E1A, is underscored by the findings that NF-Y-binding sites are also present in other cell cycle-regulated genes such as cyclin A, thymidine kinase, hsp70, and cdc25C (reviewed in Ref. 57). The presence of a single wild type CCAAT box motif of the cdc2 promoter was sufficient for transactivation by E1A 3–5-fold. However, when both CCAAT motifs were mutated, no specific DNA-protein complexes could be formed and transactivation of the cdc2 promoter by E1A was abolished (43). E1A has been shown to activate gene expression of cyclin A, thymidine kinase, and hsp70 (58–60), suggesting the possibility that NF-Y may also play a role in transcription of these cell cycle regulatory genes. Supporting this notion is the observation that a complex of a 115-kDa protein and CBF/NF-Y was shown to interact with the CCAAT motif of the cyclin A promoter and this interaction is necessary for adhesion-dependent transcription of cyclin A gene (61). However, the binding specificity of the 115-kDa protein to the CCAAT motif of cyclin A promoter has not been analyzed.

Nuclear extracts from human 293 cells specifically bind the CCAAT box motifs of cdc2, cyclin A, cdc25C, and hsp70 promoters to form the specific DNA-protein complexes (43, 62). However, there are some differences in the patterns observed in EMSA: the distal CCAAT element of the cdc2 and cdc25C promoters and the CCAAT element of the hsp70 promoter formed three specific complexes (CI to CIII). However, the cyclin A promoter element and the proximal motif of the cdc2 promoter formed only complex I (Ref. 62 and this study). The complexes II and III formed with the distal motif of the cdc2 promoter were competed out by the cold oligonucleotides containing the distal cdc2 motif, suggesting that these are specific complexes. However, they did not include NF-Y as shown by supershift EMSA assays. Further work is necessary to identify the proteins interacting with the distal CCAAT element of not only the cdc2 promoter but also the cdc25C promoter and the CCAAT element in the hsp70 promoter.

Our previous observation that the basal activity of the mutant cdc2 promoter/reporter construct in which both CCAAT motifs were mutated was increased 1.75-fold compared with the wild type construct in the absence of adenovirus E1A (43), suggested a possibility that the wild type CCAAT motifs of the cdc2 gene may in fact mediate transcriptional repression in cycling cells rather than activation in the absence of E1A. The role of E1A in the activation of the cdc2 promoter may be to overcome this repression and couple upstream transcriptional activators to the basal transcription machinery. Consistent with this notion, cell cycle-dependent element (CDE)-mediated transcriptional repression, caused by binding of proteins to CDE, has been proposed as a mechanism to explain the periodic activation of cdc25C, cyclin A, and cdc2 during the cell cycle (63). Although promoters of all three genes contained the CDE element, only cdc2 and cdc25C promoters contained the two CCAAT elements (referred as Yc-Box1 and Yc-Box2 in Ref. 63). However, the cyclin A promoter contained only one CCAAT element which was similar to the consensus sequence present in the proximal cdc2 promoter.

In vivo footprinting studies suggested that a CDE mapping at −20 of the cdc2 promoter (64) and −15 of the cdc25C promoter (65) with respect to the transcription start site, together with cell cycle homology region (CHR), negatively regulates the cdc2 gene expression (for review, see Ref. 66). CDE element is similar to the consensus E2F site except it lacks a T-rich region upstream of the G/C core. The major protein complex binding at −20 element of the cdc2 promoter is the E2F-4/p130 and between the member of E2F transcription factor family and the E1A-associated protein of retinoblastoma protein family (64). Furthermore, the E2F-4/p130 complex functioned as a repressor predominantly in quiescent cells and to some extent also in cycling cells (64). In the case of cdc25C promoter it was shown that while for the transcriptional repression via the CDE-CHR regions, the upstream activating sequence which contains recognition sites for NF-Y and Sp1, were also required (67). Therefore, the increased basal activity of the cdc2 promoter by mutation of the two CCAAT motifs (43) suggests that NF-Y may also be under negative regulation of CDE-CHR elements of the cdc2 promoter due to binding of E2F-4/p130 in the absence of E1A. The role of E1A is then to relieve this repression by multiple pathways. For example, E1A could disrupt the E2F-4/p130 complex by binding to the p130 (68), as the cells exit G1, to enter S phase. As shown in the present study, E1A also activates gene expression of all three subunits of CBF/NF-Y. The supporting evidence for the role of newly synthesized NF-Y playing an important role in E1A-mediated transactivation of the cdc2 promoter comes from our results that the dominant negative mutant NF-YA inhibits this transactivation. This inhibition could result from either interference of the dominant negative mutant with the assembly of the heterotrimeric NF-Y or interaction between NF-Y and the 110-kDa CBFA/cdc2 or both.

The functional domains of E1A protein that are required for immortalization, cellular DNA synthesis, cdc2 gene expression, and p34cdc2 kinase activity have been studied in detail by mutational analysis (22, 24, 69–71). E1A mutants defective in binding one or the other of pRB family or p300/CREB family of proteins were still capable of inducing DNA synthesis and p34CDC2 kinase activity (22, 24, 72). However, E1A mutants which lost the ability to bind both families of proteins were defective in these functions. The requirement of the E1A domains for the induction of the 110-kDa CBFA also showed a similar pattern (43).

There are several proteins which are known to bind CCAAT motifs: C/EBF/NF-1 (73), CBF/NF-Y (also known as CP1 (46, 54, 74)), CP2 (74), CBP (75), C/EBP (76), and a 114-kDa CBF which is involved in the activation of hsp70 promoter (77, 78) (referred here as CBF/HSP70). E1A.128 stimulates the transcription of the endogenous CBF/hsp70 gene (43). Moreover, the 114-kDa CBF/HSP70 also transactivated cdc2 promoter/reporter expression in transient transfection assays carried out in HeLa cells (43) but not in CV1 cells (42). These results suggest that the transactivation of the human cdc2 promoter by the ectopic expression of the CBF/HSP70 appears to depend on cell type. These observations are in accord with the previous report that the transactivation of the hsp70 promoter/reporter by CBF/HSP70 in transient transfection assays was also dependent on cell type; CBF/HSP70 was active in COS cells, which constitutively express SV40 large T antigen, but not in Chinese hamster ovary cells (79). Co-transfection of E1A and the hsp70 promoter/CAT plasmid into Chinese hamster ovary cells was necessary to transactivate the hsp70 promoter/CAT expression. We previously reported that heteromeric complex I,
which includes the CBF/NF-Y and the 110-kDa protein as shown in this study, are overexpressed in HeLa cells and COS cells. The 110-kDa/NF-Y complex from COS cell or 293 cell nuclear extracts, bound to the distal CCAAT motif of the cdc2 promoter, was not supershifted by anti-CBF/HSP70 antibody (Ref. 42, data not shown) suggesting that complex I does not include the CBF/HSP70. These observations, taken together, suggest that prior induction of the 110-kDa protein by the viral oncoproteins may be required for activation of the hsp70 promoter by the 114-kDa CBF/HSP70 protein over the levels achieved with the 110 kDa/NF-Y complex alone.

Not only the cellular genes and/or host cell cycle are affected, the ability of E1A to activate NF-Y also might have a great impact on viral life cycle. The adenovirus major late promoter, one of the most intensively studied eukaryotic polymerase II promoters, has several regulatory binding sites for transcription factors, including an inverted CCAAT box that binds to NF-Y. In a recent study (80) on functional analysis of this CCAAT box in major late promoter revealed that mutation of this region greatly reduced the viral replication. Although the CCAAT sequence can be replaced by ATF- and OCT1-binding sites without losing its function, the CCAAT motif was still a preferred site (80) and it is highly conserved among mammalian adenoviruses.

NF-Y is known to function with other accessory DNA-binding proteins, which serve as cooperative factors to achieve enhanced gene expression. Some examples are, steroid regulatory element-binding protein in the regulation of 3-hydroxy-3-methylglutaryl-coenzyme A synthase gene (49), Sp1 in the regulation of major histocompatibility complex class II-associated invariant chain gene (81) as well as the human multiple drug-resistance gene (82), RF-X, in the major histocompatibility complex class II promoter regulation expression (83), C/EBP in liver-specific albumin gene expression (48), C/EBPβ in the regulation of the human liver-specific albumin gene (84) and Tax1 protein, encoded by human T-cell lymphotropic virus type I (85), which is involved in transcriptional activation of a number of cellular genes.

For the deregulation of cell cycle-regulated cdc2 gene expression by the oncoprotein E1A, our study provides the first evidence that both the 110-kDa CBAP and CBF/NF-Y are induced which form a heteromeric complex. There is increasing evidence that aberrations in expression of cell cycle regulatory proteins such as cyclins, CKDs, and CDK inhibitors are closely linked to development and progression of carcinogenesis. De-regulation of cell cycle control is a hallmark of cancer cells (86–90). We recently reported that the 110-kDa CBAP is over-expressed in a variety of human tumor cell lines and rodent cell lines transformed by different oncopgenes or spontaneously (62). Therefore, the results presented in this study taken together with previous studies suggest that aberrant expression of the components of the heteromeric protein complex, the 110-kDa CBAP/cdc2 and NF-Y, may play an important role in carcinogenesis.

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