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Identification of a Cell-Type-Specific and E2F-Independent Mechanism for Repression of *cdc2* Transcription

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Human myeloid leukemia cells, such as HL60, U937, and THP1 cells, undergo macrophage differentiation and growth arrest following treatment with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Surprisingly, we find that growth of a significant percentage of THP1 cells is arrested in the G_2 phase of the cell cycle. G_2 arrest correlates with cell-specific repression of the gene encoding $p34^{cdc2}$, a crucial regulator of G_2/M progression. Intriguingly, TPA-mediated repression of the *cdc2* promoter was independent of the transcription factor E2F, distinguishing this pathway from mechanisms responsible for repression of *cdc2* transcription in response to serum starvation. The region of the *cdc2* promoter required for repression was located from bp −22 to −2 from the major transcriptional start site. This sequence, which we term the R box, directs the uncoupling of the basal promoter from upstream activators following TPA treatment. Analysis of THP1 nuclear proteins revealed a 55-kDa protein that was induced by TPA and interacted with the *cdc2* promoter in an R-box-dependent manner. These observations provide evidence for the existence of cell-type- and promoter-specific pathways for the assembly of stable transcriptional initiation complexes that function to differentially regulate the expression of cell cycle control genes in mammalian cells.

Coordinate regulation of cellular growth is critical for the development and homeostasis of multicellular organisms. Within the hematopoietic system, the proliferative response of different subsets of leukocytes is regulated by a diverse array of growth-promoting and growth-inhibitory factors. Differential patterns of expression of these molecules and their respective receptors permit highly specific programs of growth and differentiation by the various hematopoietic lineages. Disruption of the balance between proliferative and antiproliferative signals can lead to abnormal programs of growth associated with leukemia and other neoplastic disorders.

The cell-type-specific mechanisms by which various growth-inhibitory molecules function to influence cellular proliferation are poorly understood. The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) represents an example of a substance that can promote growth in some cell types while inhibiting growth in others (1, 7). TPA is thought to exert its effects on gene expression through activation of protein kinase C (2). Treatment of the human THP1, HL60, and U937 myeloid leukemia cell lines with TPA results in a rapid and irreversible program of growth arrest and differentiation into macrophage-like cells (for a review, see reference 7). These cell lines therefore provide model systems for examining the molecular mechanisms by which TPA inhibits growth in a manner that is dominant to the proliferative signals provided by serum growth factors.

Cell growth and proliferation are ultimately regulated by a highly conserved set of cell cycle regulatory proteins that are ubiquitously expressed in organisms from yeasts to humans (for a review, see reference 32). The G_0/G_1 transition is generally considered to be the critical interval for the integration of cues that determine either cellular proliferation or quiescence (33). NIH 3T3 fibroblasts, for example, require the presence of serum growth factors during early G_1 but are relatively insensitive to their removal for the remainder of the cell cycle (33). It is believed that serum growth factors regulate the expression and activities of a subset of G_1 cyclins and cyclin-dependent kinases (CDKs). These proteins form complexes which in turn phosphorylate specific substrates involved in regulating progression through G_1 and commitment to DNA replication (23, 27).

The retinoblastoma susceptibility gene product (Rb) is thought to represent an important substrate of the G_1 cyclin-CDKs, particularly cyclin D-CDK4 (11, 14, 23, 29, 51). The phosphorylation status of Rb is cell cycle dependent; Rb exists in a hypophosphorylated (active) state in G_0 and early G_1 and becomes hyperphosphorylated (inactivated) late in G_1. The hyperphosphorylated state is maintained through S, G_2, and most of M (4). Hypophosphorylated Rb has been demonstrated to repress the expression of growth-related genes by interacting with the transcription factor E2F (8, 20, 48). In response to growth factor stimulation, Rb becomes hyperphosphorylated and dissociates from E2F, which then functions as a positive transcription factor, promoting the transcription of growth-related genes (8, 20). These observations suggest that the phosphorylation state of Rb is a critical determinant in the execution of the progression from G_1 to S.

G_2 has been considered to be the phase of the cell cycle in which the status of DNA replication and repair is checked and the events required for the initiation of mitosis are staged (9, 33). The observations that inhibitors of DNA synthesis or damage to DNA can delay the onset of mitosis support this notion (13, 19, 33). Although checkpoints in the G_2 interval of the cell cycle may exist to ensure the integrity of the genome prior to mitosis, the G_2/M boundary has not been generally considered to be a control point for growth in higher vertebrates. Entry into mitosis requires activation of $p34^{cdc2}$-cyclin B complexes

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(12). Consistent with this, dominant negative p34^{cdc2} mutants have been shown to arrest the growth of cells in G_2/M (46). The kinase activity of p34^{cdc2} is controlled during the cell cycle both by its association with cyclin B and by phosphorylation and dephosphorylation on specific residues (12, 16, 35).

In addition to posttranslational regulation of cdc2 kinase activity, there is evidence that *cdc2* transcription is regulated in response to the growth status of the cell. Following growth arrest due to serum starvation, *cdc2* mRNA levels fall to almost undetectable levels. This situation is reversed as cells enter the cell cycle in response to growth-stimulatory signals (e.g., the addition of serum), indicating that p34^{cdc2} is regulated in a cell-cycle-dependent manner (8, 28). Analysis of the human *cdc2* promoter demonstrated that the *cdc2* gene was repressed following serum starvation and that this inhibitory effect was mediated through a binding site for E2F (8).

In this study, we have investigated the cellular and molecular events involved in the growth arrest of THP1 cells following TPA treatment. Intriguingly, growth of THP1 cells was found to be arrested not only in G_1 but also in G_2. G_2 arrest correlated with downregulation of cdc2 kinase activity, protein, and mRNA levels. Analysis of the *cdc2* promoter revealed a mechanism of transcriptional inhibition that was cell type specific and independent of the E2F binding site. The sequences responsible for repression were localized to a 21-bp sequence immediately upstream of the major transcriptional start site, suggesting that the targets of TPA include components of the core transcriptional machinery. These studies therefore provide evidence for an E2F-independent mechanism that can operate to regulate the expression of genes involved in cell cycle control in a cell-type-specific manner.

MATERIALS AND METHODS

Tissue culture. THP1, HL60, U937, and Jurkat cells (American Type Culture Collection) were cultured in RPMI 1640 medium (BioWhittaker). CV-1 cells (American Type Culture Collection) were grown in Dulbecco modified Eagle medium. Each medium was supplemented with 10% heat-inactivated fetal calf serum (Gemini), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. TPA (Sigma) was dissolved in ethanol in a 1 mM stock solution and used at 25 nM in ethanol for all experiments. Control cells were treated with an equivalent volume of ethanol alone.

Flow cytometry analysis. Cells (2×10^6 per time point) were treated with TPA for various times and were harvested by centrifugation, washed with phosphate-buffered saline (PBS) (0.137 M NaCl, 0.0054 M KCl, 0.0162 M Na_2HPO_4, 0.0029 M KH_2PO_4), and fixed in 80% ethanol. One to 3 days later, the cells were washed in PBS and resuspended in 1 ml of PBS containing DNase-free RNase (40 μg/ml) and propidium iodide (20 μg/ml; Boehringer Mannheim). After a 3-h incubation at room temperature, the cells were filtered through a 70-μm-pore-size nylon cell strainer (Falcon) and the amount of propidium iodide incorporation was determined by using a FACScan analyzer. More than 10,000 cells were scanned per time point.

cdc2 kinase assays. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer as described elsewhere (17), and the amount of protein was determined by using the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.). Total protein (100 to 200 μg) was subjected to immunoprecipitation with p34^{cdc2} antibodies as described elsewhere (25). The immunocomplexes were collected with protein A-agarose (Boehringer Mannheim) and washed twice with RIPA buffer and three times with kinase buffer (50 mM Tris [pH 7.4], 10 mM $MgCl_2$, 1 mM dithiothreitol). The kinase reactions were carried out in 25 μl of kinase buffer with 1 μg of histone H1 protein, 100 μM ATP, and 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol). The reaction mixtures were incubated at room temperature for 20 min, after which reactions were stopped by the addition of 20 μl of 5× Laemmli sample buffer (17) and mixtures were boiled for 10 min. The reaction products were separated on a 15% polyacrylamide gel, dried, and exposed to Kodak X-AR film.

cdc2 Western blot (immunoblot) analysis. Cells (5×10^6) were lysed in RIPA buffer, and the amount of protein was determined as described above. A 50-μg sample of total protein was subjected to electrophoresis on a 12% polyacrylamide gel and transferred to nitrocellulose. After blocking of the filter with 5% nonfat milk in PBST (PBS with 0.1% Tween 20) for 1 h at room temperature, the filter was washed three times in PBST and incubated for 2 h with a mouse monoclonal antibody against p34^{cdc2} (Santa Cruz Biotech). The filter was then incubated for 1 h at room temperature with a biotinylated anti-mouse immunoglobulin G, after which an amplification step with streptavidin-horseradish peroxidase complexes was carried out. The ECL system (Amersham) was used for detection, and the chemiluminescence was recorded by exposure to Kodak X-AR film for 15 s.

mRNA analysis. THP1 cells (2.5×10^7) (5×10^5 cells per ml) were treated with 25 nM TPA and harvested after various times. Poly(A)$^+$ RNA was selected from total lysates on oligo(dT) cellulose as described elsewhere (3). Five micrograms of poly(A)$^+$ RNA per lane was electrophoresed in 1% agarose gels containing 1.1 M formaldehyde, and the size-separated RNA was transferred to nitrocellulose filters. After being baked at 80°C under a vacuum for 2 h, the filters were hybridized as described previously (39). Primer extension analysis of the transcriptional start site of luciferase reporter genes was performed as previously described (31) by using an antisense luciferase primer with the sequence TGCTCTCCAGCGGTTCCATCCTCTA.

Plasmid constructions. A human *cdc2* genomic clone (kindly provided by Stephen Dalton) was cloned upstream of the firefly luciferase DNA in the expression vector Δ5′ PSV 2 Luciferase (50). Deletions at the 5′ end were made by generating oligonucleotides corresponding to *cdc2* genomic sequences and using PCR for amplification. The E2F binding site (TTTCGCGCT) located 129 bp upstream from the major transcriptional start site was mutated to TTCTC GAGT by PCR. This mutation alters 6 of the 9 bp required for high-affinity binding of E2F. The resulting wild-type and mutant promoter DNA sequences were cloned upstream of a luciferase cDNA that served as a reporter gene. 3×CCAAT and 3×PU.1 were made by annealing and concatemerizing corresponding oligonucleotides and cloning them in front of minimal *cdc2* or rat prolactin promoters driving luciferase. The sequence of the sense CCAAT oligonucleotide was 5′-ggtcagctcgaGTAGCTGGGCTCTGATTGGCTGCTagatc-3′. The sequence of the sense PU.1 oligonucleotide was 5′-gatcTGTTTCTTTT CCTTTTCACTTCTCTTTTTg-3′. Uppercase letters represent *cdc2* sequence containing and flanking the CCAAT box or PU.1 sequence, while lowercase letters represent polylinker sequence for cloning purposes. The plasmids for the chimeric promoter analysis were made by synthesizing oligonucleotides corresponding to either the *cdc2* promoter, prolactin, or chimeras from positions −36 to +25 (sequences are listed in Fig. 6) and cloned between 3× CCAAT and the luciferase reporter gene. All plasmid sequences were confirmed by dideoxy DNA sequence analysis.

Transfection studies. Logarithmically growing cells (2×10^7) were collected by centrifugation, washed in STBS (137 mM NaCl, 24 mM Tris-Cl [pH 7.4], 0.6 mM Na_2HPO_4, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$), resuspended in 800 μl of STBS containing 200 μg of DEAE dextran per ml and 5 μg of reporter DNA (CsCl gradient double banded), and incubated for 20 min at 37°C. The cells were then washed three times in STBS, resuspended in complete media, and distributed into four 10-cm-diameter plates (10 ml per plate). The cells were allowed to recover for 8 to 12 h and were then treated (in duplicate) either with 25 nM TPA or with an equal volume of solvent (ethanol) and harvested 12 h later in 50 μl of 25 mM Tris (pH 7.8)–2 mM dithiothreitol–2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid–10% glycerol–1% Triton X-100. A 20-μl volume of this lysate was used in a luciferase assay as previously described (10), except that the luciferase buffer contained *n*-butyl coenzyme A (Promega). Luciferase activity was normalized to total protein levels. All plasmid constructions were tested by transfection at least three times.

Preparation of nuclear extracts and DNA binding assays. Sense and antisense oligonucleotides corresponding to either the *cdc2* promoter, prolactin, or chimeras from positions −36 to +25 (sequences listed in Fig. 6) were synthesized on an Applied Biosystems model 394 oligonucleotide synthesizer so as to contain biotin residues at the 5′ end, which was achieved by utilizing a biotin phosphoramidite reagent according to the manufacturer's instructions. The final trityl group was retained on the cleaved product to facilitate purification of full-length oligonucleotides. The tritylated, single-stranded oligonucleotides were purified on Nensorb prep cartridges. Double-stranded oligonucleotides were prepared by annealing the sense and antisense strands at equal molar ratios. Nuclear extracts were prepared from U937 cells as previously described (50), with the additional step of labeling cellular proteins with [^{35}S]methionine and [^{35}S]cysteine for 8 h prior to extract preparation. Nuclear extracts (1 mg per time point) were precleared by incubation with 50 μl of streptavidin-agarose beads (Pierce) for 1 h at 4°C with continuous agitation. The beads were removed by centrifugation, and the supernatants were subjected to a second preclearing step. The precleared nuclear extracts were then incubated with 5 μg of biotinylated oligonucleotide and 50 μl of streptavidin-agarose for 1 h at 4°C with constant agitation. The preclearing and binding steps were performed in a volume of 500 μl containing 150 mM KCl, 1 mM EDTA, and 20 mM Tris, pH 8.0. Following performance of the binding reactions, the streptavidin-agarose beads were washed three times in 150 mM NaCl–1 mM EDTA–20 mM Tris, pH 8.0, and boiled in sodium dodecyl sulfate (SDS) sample buffer, and the DNA-associated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide gels).

RESULTS

TPA treatment of THP1 cells leads to cell cycle arrest in both G_1 and G_2. Treatment of THP1 cells with TPA results in an abrupt arrest of cell growth and the execution of a program

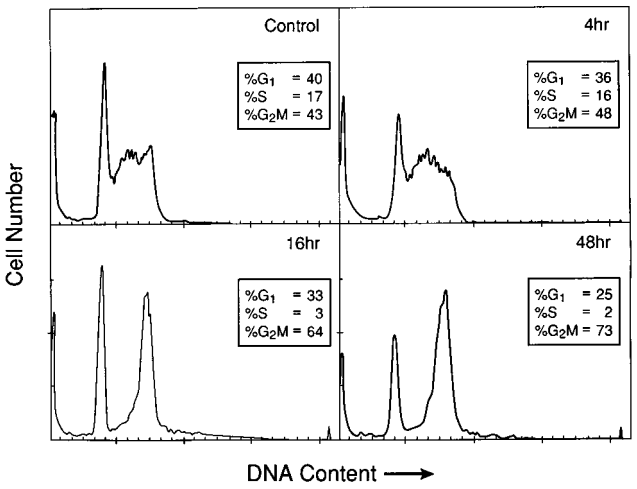

FIG. 1. TPA treatment leads to cell cycle arrest in G_1 and G_2/M. THP1 cells were treated with solvent alone (0 h) or 25 nM TPA for the indicated times and analyzed by flow cytometry. The leftmost peak comprises cells with 2 N DNA content and represents cells in G_1. The rightmost peak comprises cells with a doubled (4 N) DNA content and represents cells in G_2/M. The cells between the peaks have intermediate DNA contents and are in S phase.

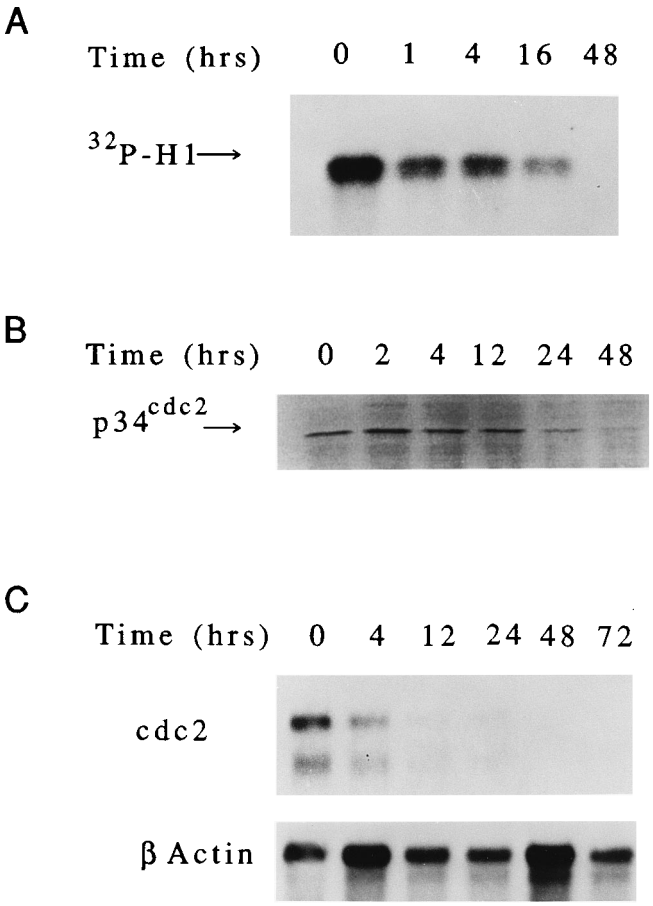

FIG. 2. Effects of TPA on $p34^{cdc2}$ kinase activity and expression. THP1 cells were treated for the indicated times. (A) Cells were collected and lysed, and equal amounts of protein were immunoprecipitated with anti-cdc2 antibodies. Immunoprecipitates were used to phosphorylate histone H1 protein. TPA inhibits histone H1 kinase activity in THP1 cells. (B) $p34^{cdc2}$ was immunoblotted as described in Materials and Methods. $p34^{cdc2}$ protein levels are downregulated after TPA treatment. (C) *cdc2* mRNA was analyzed by Northern blot analysis. *cdc2* mRNA is downregulated after TPA treatment in THP1 cells. After cdc2 hybridization, the blot was stripped and rehybridized with a probe specific for β-actin to assess the relative amounts of RNA loaded in each lane.

of macrophage differentiation (30, 44). To investigate the mechanisms responsible for growth arrest, an analysis of the distribution of cells in the various phases of the cell cycle was performed by flow cytometry as a function of time following TPA treatment (Fig. 1). Untreated, logarithmically growing THP1 cells were distributed as follows: 40% were in G_1, 17% were in S, and 43% were in G_2/M. By 4 h after TPA treatment, this profile had not significantly changed, but by 24 h the percentage of cells in S phase had dropped more than fivefold, to 3%. Consistent with these results, thymidine incorporation assays indicated that DNA synthesis was inhibited by more than 90% in TPA-treated THP1 cells (data not shown). Remarkably, TPA treatment also caused a progressive increase in cells whose growth was arrested at G_2/M. By 48 h, the percentage of cells in G_2 had increased to 73% while the percentage of cells in G_1 decreased to 25%. Microscopic analysis of these cells indicated that very few (<1%) had entered M phase, as evidenced by preservation of nuclear architecture and an interphase pattern of chromatin staining revealed by using intercalating dyes (data not shown). Thus, treatment of THP1 cells with TPA resulted in a G_2/M block and, to a lesser extent, a block in G_1. Although less pronounced, growth of a significant percentage of cells was also found to be arrested at the G_2/M boundary when HL60 or U937 cells were induced to differentiate into macrophage-like cells with TPA (data not shown), indicating that the G_2/M arrest was not restricted to THP1 cells. Nonmyeloid cell types, such as the Jurkat T-cell line, did not display this alteration in their cell cycle profile in response to TPA (data not shown).

TPA treatment downregulates $p34^{cdc2}$ kinase activity, protein, and mRNA levels. The observation that TPA induced growth arrest at both G_1 and G_2/M boundaries suggested that multiple genes involved in cell cycle progression might be targets for regulation. Because Rb is thought to be a crucial regulator of the G_1/S transition, we first analyzed its phosphorylation status in response to treatment of THP1 cells with TPA. This analysis revealed a transition from a predominantly hyperphosphorylated form in untreated THP1 cells to a hypophosphorylated form after TPA treatment (data not shown), consistent with results of previous studies with HL60 and U937 cells (6). Thus, the effects of TPA on growth arrest in G_1 could be accounted for, at least in part, by the regulation of the phosphorylation state of Rb.

To examine potential mechanisms involved in the growth arrest at the G_2/M boundary, an analysis of $p34^{cdc2}$ kinase activity was performed. Extracts from TPA-treated THP1 cells were incubated with anti-cdc2 antibodies, and the resulting immunoprecipitates were assayed for their ability to phosphorylate histone H1 in vitro. $p34^{cdc2}$ kinase activity was reduced as early as 1 h following TPA treatment. Between 4 and 16 h, there was a pronounced drop in kinase activity, and by 48 h, kinase activity was nearly undetectable (Fig. 2A).

In order to examine whether the downregulation of kinase activity could be accounted for by downregulation of the levels of $p34^{cdc2}$ protein, Western blotting of extracts from TPA-treated THP1 cells was performed. $p34^{cdc2}$ protein levels were observed to decrease in a time course similar to that of cdc2 kinase activity, and by 48 h cdc2 protein levels were barely detectable (Fig. 2B). These observations suggested that the loss of $p34^{cdc2}$ kinase activity in response to TPA was due, at least in part, to downregulation of $p34^{cdc2}$ protein levels.

To investigate whether decreased $p34^{cdc2}$ protein levels might reflect decreased mRNA levels, Northern (RNA) blot analysis was performed. We found that mRNA levels for cdc2 were downregulated as early as 4 h after TPA treatment. By 24 h, mRNA levels for cdc2 were barely detectable (Fig. 2C, top). This effect was not due to a toxic or nonspecific effect on gene expression because the expression of the β-actin gene was unchanged following TPA treatment (Fig. 2C, bottom). In addition, the expression of MAC-1, a differentiation marker for myeloid cells, was upregulated after TPA treatment (data not shown).

TPA inhibits transcription from the *cdc2* promoter by an E2F-independent mechanism. On the basis of the marked decrease in cdc2 mRNA, transcription from the *cdc2* promoter was examined as a possible target of the actions of TPA. To analyze the transcriptional control of the *cdc2* promoter, a genomic clone of *cdc2* extending from bp −3200 to +75 from the major transcriptional start site was fused to the firefly luciferase gene and transiently transfected into THP1 cells. The transfected cells were then treated with TPA and harvested after 12 h. *cdc2* promoter activity was inhibited by approximately 80% in THP1 cells 12 h following TPA treatment (Fig. 3A). A 5′ deletion to bp −245 increased expression by approximately twofold, but negative regulation in response to TPA was unaffected. On the basis of the observation that Rb became hypophosphorylated following TPA treatment, it was expected that the inhibition of *cdc2* promoter activity would be mediated by the E2F binding site located at bp −129 from the major transcriptional start site. Surprisingly, deletions to bp −114, −104, and −92, which remove the E2F binding site, had no effect on either basal expression or regulation in response to TPA. A further deletion to bp −80, which interrupted a CCAAT box, resulted in a dramatic decrease in basal activity and a near-complete loss of negative regulation in response to TPA (Fig. 3A).

These transient transfection results were confirmed by using stably transfected cell lines. Pools of THP1 cell lines expressing luciferase under the control of either the wild-type *cdc2* promoter (positions −245 to +75) or a *cdc2* promoter containing a clustered point mutation in the E2F binding site were generated. As a control, a pool of stably transfected THP1 cells expressing luciferase under the control of the β-actin promoter was made. TPA treatment resulted in an 8- to 10-fold repression of both the wild-type *cdc2* promoter and the E2F mutant promoter (Fig. 3B). The β-actin promoter, which is not regulated by TPA (31), directed a constant level of luciferase activity, indicating that the effects of TPA on the *cdc2* promoter were promoter specific and did not reflect posttranscriptional effects on the luciferase reporter mRNA. To be certain that transcription was initiated from the appropriate locations within the *cdc2* promoter, primer extension experiments were performed with total RNA isolated from control and TPA-treated stable cell lines (Fig. 4). These experiments confirmed that the majority of *cdc2*-luciferase gene transcripts were initiated from the previously documented major transcriptional start site of the *cdc2* promoter and further demonstrated that the levels of these transcripts were downregulated in response to TPA.

Analysis of other cell types indicated that the inhibitory effect of TPA on *cdc2* promoter activity correlated with its effects on cell growth. TPA inhibited *cdc2* promoter activity in HL60 and U937 cells, whose growth is arrested in response to TPA. In addition, the downregulation of the *cdc2* promoter in HL60 and U937 cells in response to TPA was independent of the E2F site as determined by comparisons of the wild-type promoter and the *cdc2* promoter containing a mutated E2F

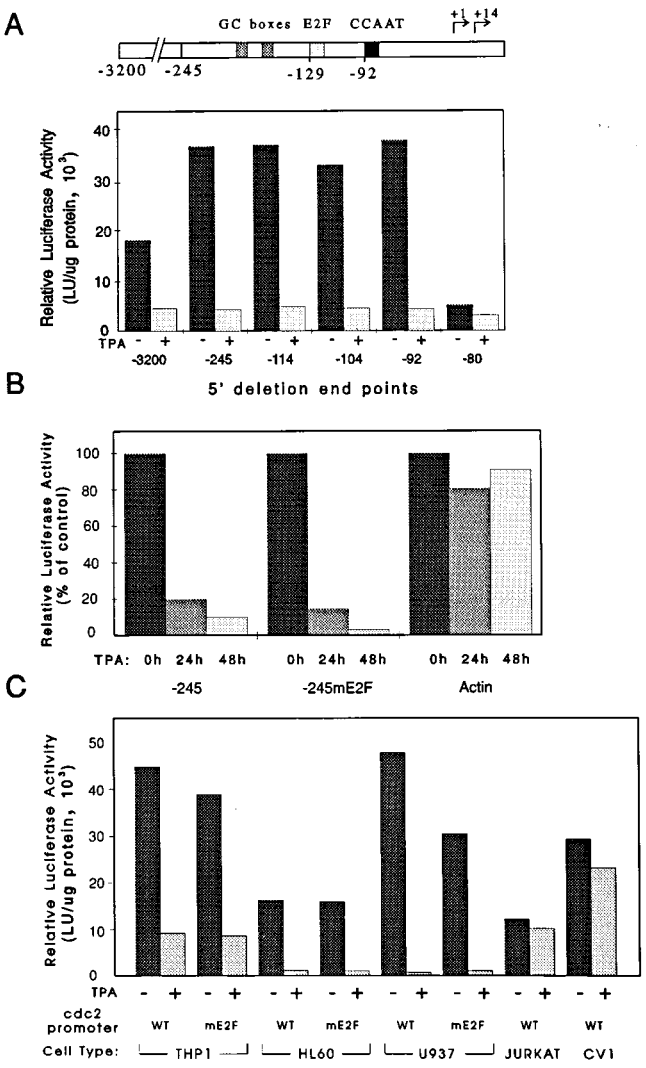

FIG. 3. TPA inhibits *cdc2* promoter activity by a mechanism that is cell type specific and E2F independent. (A) Illustration of DNA recognition motifs within the *cdc2* promoter and 5′ deletion analysis of the *cdc2* promoter in THP1 cells. THP1 cells were transfected with plasmids containing *cdc2* promoters extending from the indicated 5′ end to bp +75 relative to the transcriptional start site. Transcriptional activity was assayed by using a luciferase reporter gene. (B) Negative regulation of the *cdc2* promoter by TPA is independent of the E2F site in stable THP1 cell lines. THP1 cells were stably transfected with reporter plasmids in which luciferase expression was directed by either the wild-type *cdc2* promoter (positions −245 to +75; left), a *cdc2* promoter containing a cluster mutation in the E2F site at position −129 (−245mE2F; middle), or the β-actin promoter (right). Pools of stable transformants were treated with TPA, harvested, and assayed at the indicated times. (C) Inhibition of the *cdc2* promoter is cell type specific. The indicated cell types were transiently transfected with either the wild-type (WT) or E2F mutant (mE2F) *cdc2* promoter fused to the luciferase gene. LU, light units.

site (Fig. 3C). TPA had no effect on *cdc2* promoter activity in the Jurkat T-cell line or CV-1 cells, whose growth is not arrested in response to TPA (Fig. 3C). The marked inhibitory effect of TPA on *cdc2* promoter activity in THP1, HL60, and U937 cells was consistent with a mechanism in which the downregulation of cdc2 protein and mRNA levels reflected repression of *cdc2* gene transcription. In concert, these experiments suggest a cell-type-specific mechanism for transcriptional inhibition of the *cdc2* promoter that was independent of E2F.

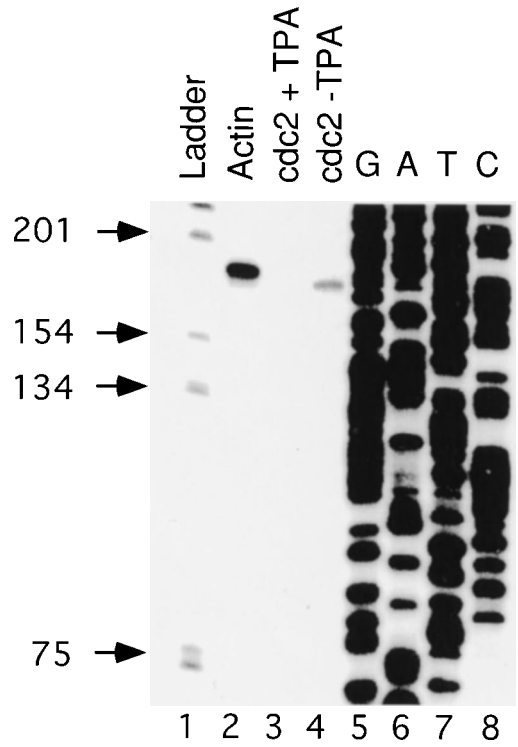

FIG. 4. Primer extension analysis of β-actin–luciferase gene and *cdc2*-luciferase gene transcripts in stably transfected THP1 cells. Total mRNA was prepared from THP1 cells stably transfected with either the β-actin–luciferase gene (lane 2) or the −245 *cdc2*-luciferase gene (lanes 3 and 4). Cells were treated with either TPA (lane 3) or solvent (lane 4) for 24 h prior to the isolation of mRNA. Reverse transcription reaction mixtures were primed with a ^{32}P-labeled luciferase primer and analyzed by denaturing polyacrylamide gel electrophoresis. The luciferase primer was used for parallel sequencing reactions of the −245 *cdc2*-luciferase gene reporter plasmid to determine the precise transcriptional start sites (lanes 5 to 8). Short exposures of the sequencing reaction products indicated a major transcriptional start site identical to that previously reported by Dalton (8). Transcription from this site was markedly downregulated following TPA treatment (compare lanes 3 and 4). Numbers on the left indicate molecular sizes in base pairs.

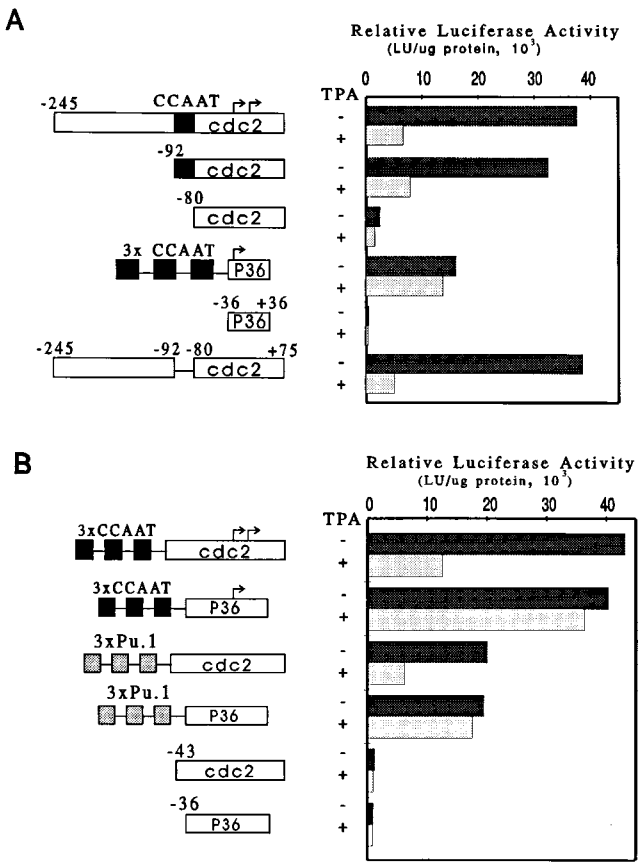

FIG. 5. (A) Activators are not the target of negative regulation by TPA. Transient transfection assays were performed as described in the legend to Fig. 3 and Materials and Methods. Lane 1, wild-type *cdc2* (positions −245 to +75); lane 2, deletion of upstream sequences to position −92; lane 3, deletion of CCAAT box (to position −80); lane 4, transfer of three copies of the CCAAT box to the prolactin promoter (positions −36 to +36); lane 5, the minimal prolactin promoter alone (positions −36 to +36); lane 6, specific deletion of the CCAAT box in the context of the *cdc2* promoter to position −245. (B) The PU.1 transcription factor can downregulate the *cdc2* promoter but not the prolactin promoter. Lane 1, 3×CCAAT driving *cdc2* (positions −43 to +75); lane 2, 3×CCAAT driving the minimal prolactin promoter (P36); lane 3, 3×PU.1 driving *cdc2* (positions −43 to +75); lane 4, 3×PU.1 driving P36; lane 5, the minimal *cdc2* promoter (positions −43 to +75); lane 6, the minimal prolactin promoter (P36). LU, light units.

Upstream activators are not the targets of negative regulation by TPA. The deletion analysis of the *cdc2* promoter suggested that the CCAAT box sequence, present in the antisense strand at position −80, might represent the target of negative regulation by TPA. Electrophoretic mobility shift assays with radiolabeled oligonucleotides corresponding to the *cdc2* promoter sequence from positions −92 to −65 revealed the presence of a single protein-DNA complex that required the CCAAT box and approximately 4 additional bp of flanking sequence for high-affinity binding. No significant changes in the binding or migration pattern of this protein were observed following TPA treatment (data not shown).

To determine whether the CCAAT box sequence could transfer negative regulation to a heterologous promoter, three copies of the *cdc2* CCAAT box element (3×CCAAT) were transferred to a minimal heterologous promoter derived from the rat prolactin gene. The presence of this element enhanced transcription from the minimal promoter approximately 30-fold but did not confer negative regulation in response to TPA (Fig. 5A, 3×CCAAT-P36). Finally, specific deletion of the *cdc2* CCAAT box in the context of the wild-type (positions −245 to +75) *cdc2* promoter had no effect on basal expression or downregulation in response to TPA (Fig. 5A). These observations indicated a redundancy in the utilization of upstream transcription factors required for the expression of the *cdc2* promoter and suggested that negative regulation by TPA did not depend on a specific upstream activator. To test this idea further, three copies of an oligonucleotide containing the binding site for the B-cell- and macrophage-specific transcription factor PU.1 (3×PU.1) were introduced upstream of a minimal *cdc2* promoter extending from bp −41 to +75 from the major transcriptional start site. In the absence of an upstream activator sequence, the basal expression level of the *cdc2* minimal promoter, while approximately eightfold above background, was not significantly regulated by TPA (Fig. 5B, −43 cdc2). The addition of PU.1 binding sites to the *cdc2* minimal promoter resulted in a significant increase in basal activity and restored negative regulation in response to TPA (3×PU.1 −cdc2 [Fig. 5B]). In contrast, when three copies of the PU.1 recognition sequence were introduced at a location proximal to the minimal prolactin promoter, they conferred a marked enhancer effect that was not subject to TPA regulation (3×PU.1 −P36 [Fig. 5B]). These observations established two important

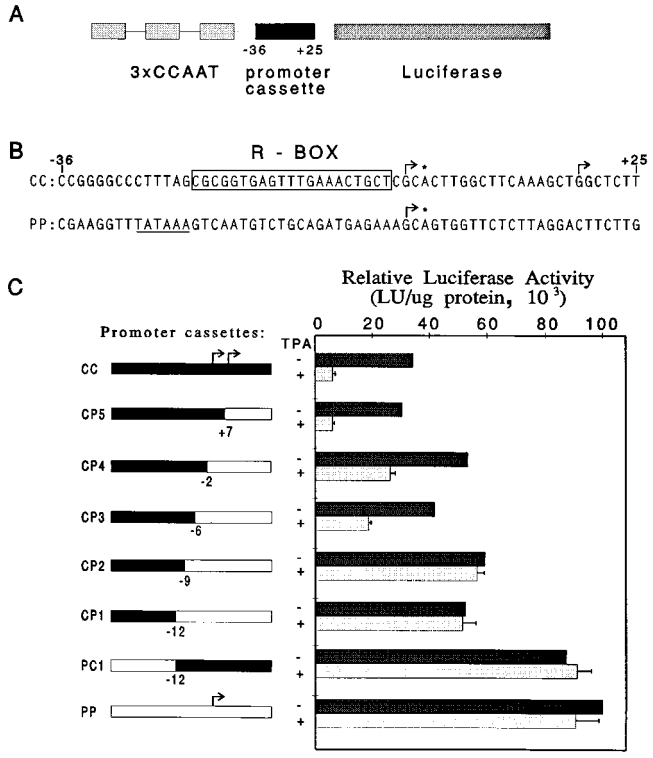

FIG. 6. *cdc2* promoter sequences mediating negative regulation by TPA in THP1 cells. (A) Diagram of reporter constructions. Three copies of CCAAT are cloned in front of a promoter cassette from positions −36 to +25 containing *cdc2*, prolactin, or chimeric promoter sequences which is fused to the luciferase reporter gene. (B) Wild-type *cdc2* and prolactin promoter cassette sequences. Arrows denote the start sites reported in the literature. Asterisks denote the start sites predicted on the basis of transcription from consensus initiator sequences. (C) The constructions indicated were transfected into THP1 cells and treated with TPA as described in the legend to Fig. 3 and Materials and Methods. Error bars represent standard deviations. LU, light units.

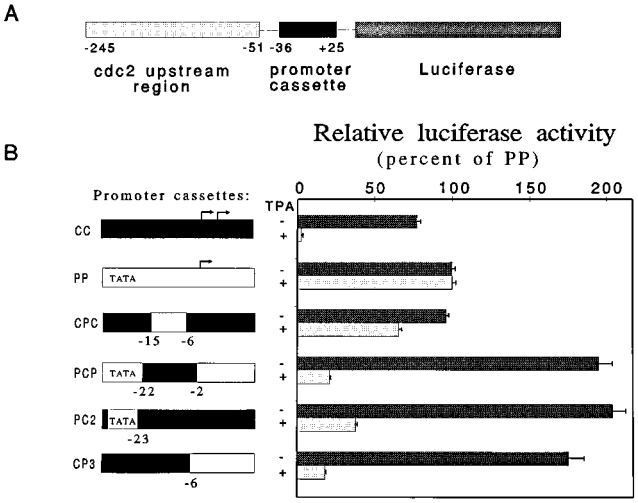

FIG. 7. Sequences between positions −2 and −22 of *cdc2* (the R box) mediate negative regulation. (A) Diagram of reporter constructions. *cdc2* upstream sequences from positions −245 to −51 were cloned in front of a promoter cassette and fused to the luciferase reporter gene as described in the legend to Fig. 6. (B) The indicated constructions were transfected into U937 cells and treated with TPA as described in the legend to Fig. 3 and Materials and Methods. Error bars represent standard deviations.

points. First, the minimal *cdc2* promoter was not itself negatively regulated by TPA in the absence of an upstream activator sequence. Second, the transcription factors binding to upstream sequences of the *cdc2* promoter were not themselves the targets of negative regulation. This suggested that TPA functioned to uncouple the actions of upstream activators from the core transcriptional machinery in a promoter-specific manner.

Identification of promoter-specific sequences required for negative regulation by TPA. To identify the minimal sequences required for promoter-specific negative regulation in response to TPA, a series of 3′ and 5′ deletions of the *cdc2* promoter was performed. These studies defined the region between bp −36 and +25 from the major transcriptional start site to be sufficient to mediate negative regulation in response to TPA when linked to an upstream activator sequence (data not shown). On the basis of the results of these studies, chimeric promoters in which sequences from the minimal *cdc2* promoter were replaced with the corresponding regions of the minimal prolactin promoter were made. Expression from these promoters was driven by three upstream CCAAT box sequences (Fig. 6A and B). The minimal *cdc2* promoter was negatively regulated by approximately fivefold in response to TPA, while expression from the minimal prolactin promoter was not affected by TPA (Fig. 6C, CC and PP, respectively).

We next examined a series of promoter chimeras in which progressive exchanges of *cdc2* and prolactin sequences were made at the 3′ end of the *cdc2* promoter. Exchange of sequences downstream of the major transcriptional start site of *cdc2* with the corresponding region from the prolactin promoter had no effect on basal or regulated expression (Fig. 6C, CP5). Exchanges made at position −2 or −6, which transfer the initiator (INR) sequences of the prolactin promoter, reduced, but did not abolish, negative regulation in response to TPA in THP1 cells (Fig. 6C, CP4 and CP3, respectively). Negative regulation was abolished by extension of the transferred region to position −9 or −12 (Fig. 6C, CP2 and CP1, respectively). We next examined promoter chimeras containing exchanges of *cdc2* and prolactin sequences at the 5′ end of the *cdc2* promoter. Negative regulation was also abolished by a 5′ replacement of *cdc2* sequence with the prolactin sequences from positions −36 to −12 (Fig. 6C, PC1).

Chimeric *cdc2* and prolactin promoters were also transfected into U937 cells in order to confirm the results obtained with THP1 cells with an independent cell line in which cdc2 was negatively regulated in response to TPA. In this cell type, the CCAAT boxes were less effective as upstream activator sequences. Therefore, the *cdc2* promoter sequence from bp −245 to −51 from the transcriptional start site was used to drive expression from the minimal *cdc2* and prolactin promoters in these cells (Fig. 7A). Consistent with the results obtained with THP1 cells, transcription from the *cdc2* promoter was strongly inhibited in U937 cells following TPA treatment (Fig. 7B). A 3′ replacement of *cdc2* sequence with prolactin sequence to position −2, while reducing the amount of repression following TPA treatment in THP1 cells, had no effect on negative regulation in U937 cells, demonstrating that the INR did not play a role in negative regulation in these cells (Fig. 7B, CP3). Furthermore, an exchange made from positions −23 to −28, which transferred the TATA box of the prolactin promoter to *cdc2*, also had no effect on negative regulation in U937 cells (Fig. 7B, PC2). These observations suggested that negative regulation in response to TPA was dependent on sequences residing between the TATA and INR regions of

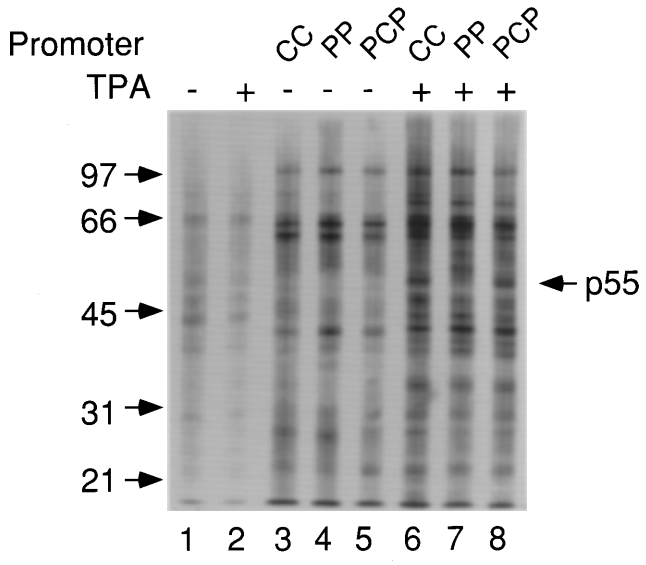

FIG. 8. A 55-kDa protein that binds to the *cdc2* promoter in an R-box-dependent manner is induced in response to TPA. Double-stranded oligonucleotides corresponding to the *cdc2* promoter from positions −36 to +25 (CC), the prolactin promoter from positions −36 to +25 (PP), and the prolactin promoter containing the *cdc2* R box (PCP) were prepared. The oligonucleotides were synthesized so as to contain biotin residues at the 5′ ends. Biotinylated promoters were incubated with nuclear proteins isolated from control or TPA-treated cells grown for 8 h in the presence of [^{35}S]cysteine and [^{35}S]methionine. Protein-DNA complexes were captured with streptavidin-agarose beads, washed, and analyzed by SDS-polyacrylamide gel electrophoresis. To control for nonspecific binding of proteins to streptavidin-agarose, incubations were performed with nuclear proteins in the absence of the biotinylated promoters (lanes 1 and 2). TPA treatment resulted in the induction of several DNA binding activities. One of these corresponded to a 55-kDa protein that bound to CC and PCP, but not PP, indicating a requirement for the *cdc2* R box. Numbers on the left indicate molecular mass in kilodaltons.

cdc2. We therefore constructed a chimeric promoter in which the *cdc2* sequences from positions −15 to −6 were replaced with the corresponding regions from the prolactin promoter. The resulting chimera regulated only minimally in response to TPA in U937 cells (Fig. 7B, CPC). The importance of the region between the TATA motif and the INR was further supported by the analysis of a reciprocal exchange in which prolactin sequences between positions −2 and −22 were replaced with the corresponding regions from the *cdc2* promoter. This sequence, which is designated the R box (for regulatory) (Fig. 6B), transferred negative regulation to the prolactin promoter (Fig. 7B, PCP) and therefore defined the sequence element that mediated promoter-specific negative regulation by TPA.

To examine the possibility that the R box was recognized by a discrete, sequence-specific DNA-binding protein that functioned to inhibit the binding of the core transcriptional machinery, electrophoretic mobility shift assays and DNase I footprinting studies were performed under a broad range of conditions that permitted high-affinity binding of several classes of sequence-specific transcription factors to their cognate recognition sequences. These studies failed to demonstrate a clear difference in the overall migration or footprinting patterns of the multiprotein complexes that bound to each promoter (data not shown). We therefore developed an assay in which metabolically labeled proteins were allowed to interact with biotinylated promoter sequences. Protein-DNA complexes were then captured on streptavidin-agarose beads and washed, and the DNA-associated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 8). Three biotinylated promoter sequences were examined, the wild-type *cdc2* promoter (Fig. 8, CC), the wild-type prolactin promoter (PP), and the prolactin promoter chimera containing the *cdc2* R box (PCP). With metabolically labeled nuclear extracts obtained from control U937 cells, similar patterns of proteins were observed to bind to CC, PP, and PCP (Fig. 8, lanes 3 to 5). One of the major proteins, with an apparent molecular mass of 37 kDa, comigrated with in vitro-translated TATA binding protein (data not shown). TPA treatment resulted in an increase in the relative levels of several bound proteins, the majority of which interacted equivalently with CC, PP, and PCP. However, one of the induced proteins, with an apparent molecular mass of 55 kDa (p55), interacted preferentially with the CC and PCP promoters, while a slightly larger protein (p57) interacted preferentially with the PP promoter (Fig. 8, lanes 6 to 8). p55 thus represents a protein that is induced by TPA and is R box specific.

DISCUSSION

Implications of G_2 arrest. A large body of evidence supports the idea that cellular decisions determining proliferation or quiescence are made during the G_1 interval (33). G_1 cyclin-CDK complexes appear to represent important cellular links between external signals, such as growth factors, and the commitment to DNA synthesis. The activities of these CDKs appear to be regulated at multiple levels, including transcriptional regulation of the G_1 cyclins, posttranslational control of cyclin-CDK activities through phosphorylation, and interactions with inhibitor proteins (18, 26, 27, 36). Although regulated by a similar and perhaps overlapping set of cyclins and CDKs, the G_2/M transition has not been a focal point in studies elaborating the mechanisms of growth control in higher vertebrate systems (9, 33). Growth arrest stemming from a G_2/M block is unusual in vertebrate cells, perhaps because an apoptotic program ensues, eliminating cells that fail a G_2 checkpoint. The observation that the majority of THP1 cells arrest their growth in the G_2 phase of the cell cycle was, therefore, surprising. It has recently been demonstrated that activation of cdc2 is required for apoptosis in YAC-1 lymphoma cells (40). Downregulation of cdc2 in THP1 cells following TPA treatment would therefore be consistent with an abnormal or disrupted apoptotic program permitting a relatively large number of cells to be arrested at the G_2/M boundary. Such an abnormal program of apoptosis may contribute to the leukemic phenotype, as has been demonstrated for chronic lymphocytic leukemias containing chromosomal rearrangements (43).

A cell-type-specific and E2F-independent pathway for inhibition of cdc2. The THP1, HL60, and U937 myeloid leukemia cell lines rapidly and dramatically arrest their growth in response to TPA and downregulate *cdc2* promoter activity. In contrast, *cdc2* promoter activity is not downregulated in cell lines whose growth is not arrested in response to TPA, such as the Jurkat T-cell line and CV-1 cells. Because the E2F site in the *cdc2* promoter is not required for TPA-dependent negative regulation in THP1, HL60, and U937 cells, these studies have defined an E2F-independent pathway that functions in a cell-specific manner to inhibit *cdc2* transcription. These observations may be relevant for other cell cycle regulatory genes as well. For example, cyclins A, B, and E, as well as CDK2, are also downregulated in response to TPA (42a). The mechanisms responsible for the downregulation of other cell cycle control genes remain to be established.

Mechanisms of R-box-mediated repression. Negative regulation of the *cdc2* promoter was dependent on both upstream

activator sequences and a sequence between the INR and the TATA motifs that we have termed the R box. The transcription factors binding to the upstream activator sequences were not the direct targets of negative regulation, because their ability to enhance expression from other promoters was not influenced by TPA. The minimal *cdc2* promoter was not significantly inhibited by TPA in the absence of upstream activator sequences, indicating that the R box alone was not capable of mediating repression of basal transcription. However, when an upstream activator sequence and the R-box sequence were present together, activated transcription was repressed following TPA treatment. These results suggest a model in which inhibition of *cdc2* transcription by TPA reflects a promoter-specific uncoupling of upstream activators from the basal transcriptional machinery.

Upstream activators are thought to stimulate transcription by interacting with components of the core transcriptional machinery (37). Recognition of the TATA motif is mediated by the TFIID complex (for a review of Pol II transcription, see reference 52). This complex includes the TATA-binding protein (TBP) and TBP-associated factors (TAFs). TAFs have been shown to interact directly with upstream activators; for example, SP1 and VP16 interact with dTAF110 and dTAF40, respectively (15, 21, 37). In promoters lacking a TATA box, an INR element appears to direct TFIID to the start site (22, 24, 42, 49). Footprinting studies indicate that the TFIID complex interacts with an extended sequence of DNA surrounding the start site. In addition to the interactions between TBP and the TATA motif, protein-DNA contacts have been described for dTAF150 between positions −1 and +38, approximately (47). These interactions of components of the core transcriptional machinery with the promoter may be modified by additional sequence-specific binding proteins. For example, YY1 binds to the adenovirus-associated P5 promoter at the INR and has been shown to repress transcription from this promoter in the absence of E1A (41). YY1, in combination with TFIIB, has also been shown to direct transcription from the P5 promoter in the absence of TBP (45). In addition, TFII-I has been shown to bind the initiator sequence of the adenovirus major late promoter and stimulate basal transcription, replacing TFIIA and stabilizing TBP binding (38). These examples support the idea that distinct complexes of core factors can assemble and direct transcription in a promoter-specific fashion, providing an additional layer of transcriptional regulation.

On the basis of these considerations, two general mechanisms could account for negative regulation of the *cdc2* promoter by TPA. In the first case, a cell-type-specific protein that is induced or activated by TPA would bind to the sequence between positions −2 and −22 and function as a classical repressor, by preventing the core transcriptional machinery from forming a functional preinitiation complex. If this model is correct, it should be possible to define this DNA binding activity biochemically. However, attempts thus far to identify a DNA-binding protein that exhibits the appropriate pattern of sequence specificity and prevents the binding of core factors have yielded only negative data. The observation that *cdc2* basal transcription is not repressed by TPA also argues against a model involving a classical repressor, because in that scenario, both basal and enhanced expression would be inhibited.

In the second general case, the target of TPA would be a component of the core transcriptional machinery involved in the recognition of the R box. This mechanism would be consistent with the existence of distinct cell-type- and promoter-specific pathways for the formation of a stable transcriptional complex (for example, see references 5, 14a, and 34). Such pathways could involve the differential utilization of cell-specific basal factors that discriminate the R-box sequence. Alternatively, the R-box sequence could influence the order of assembly or the conformation of a common set of basal factors, which would permit TPA to uncouple the resulting complex from upstream activators. To date, there have been no proven examples of promoter-specific TAFs. However, relatively few cell types and promoters have been examined in detail with respect to this issue.

By using biotinylated promoters to capture protein-DNA complexes, a 55-kDa protein was identified that was induced in U937 cells following TPA treatment and bound selectively to regulated promoters (i.e., CC and PCP) in an R-box-dependent manner. This protein is therefore likely to play a role in R-box-mediated repression of the *cdc2* promoter following TPA treatment. A 57-kDa protein was induced that bound to the prolactin promoter but not the *cdc2* promoter. It is not yet clear whether p55 and p57 are distinct proteins or whether they represent the same protein that has been differentially phosphorylated or otherwise modified. It will be of considerable interest to determine the identities of p55 and p57 and further clarify their roles in regulating promoter function.

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