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Mechanisms of G2 Arrest in Response to Overexpression of p53

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Overexpression of p53 causes G2 arrest, attributable in part to the loss of CDC2 activity. Transcription of *cdc2* and *cyclin B1*, determined using reporter constructs driven by the two promoters, was suppressed in response to the induction of p53. Suppression requires the regions −287 to −123 of the *cyclin B1* promoter and −104 to −74 of the *cdc2* promoter. p53 did not affect the inhibitory phosphorylations of CDC2 at threonine 14 or tyrosine 15 or the activity of the cyclin-dependent kinase that activates CDC2 by phosphorylating it at threonine 161. Overexpression of p53 may also interfere with the accumulation of CDC2/cyclin B1 in the nucleus, required for cells to enter mitosis. Constitutive expression of cyclin B1, alone or in combination with the constitutively active CDC2 protein T14A Y15F, did not reverse p53-dependent G2 arrest. However, targeting cyclin B1 to the nucleus in cells also expressing CDC2 T14A Y15F did overcome this arrest. It is likely that several distinct pathways contribute to p53-dependent G2 arrest.

INTRODUCTION

The p53 tumor suppressor helps to protect mammals from developing neoplasia by blocking cell cycle progression or by inducing cell death in response to stress (Ko and Prives, 1996; Levine, 1997; Agarwal *et al.*, 1998b). p53-mediated arrest at the G1–S boundary prevents the replication of DNA damaged by ionizing or UV radiation or by chemical mutagens (Kastan *et al.*, 1991; Gujuluva *et al.*, 1994; Ceraline *et al.*, 1998). G1 arrest depends on the ability of p53 to activate the transcription of specific genes (Dulic *et al.*, 1994; Pietenpol *et al.*, 1994). An important target, p21/waf1, inhibits cyclin-dependent kinases (CDKs)[1] 2, 4, and 6, which are required to enter S phase (El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993). p53 also inhibits S-phase entry in response to damage to the mitotic spindle, preventing the rereplication of DNA (Cross *et al.*, 1995), possibly by a transcription-independent mechanism (Notterman *et al.*, 1998). p53 mediates G1 arrest in response to nucleotide deprivation, preventing DNA synthesis under conditions that would generate damaged DNA (Chernova *et al.*, 1995; Linke *et al.*, 1996). p53 also protects cells arrested within S phase by a lack of pyrimidine nucleotides, preventing the replication from unbalanced pools of deoxynucleoside triphosphates and consequent DNA damage (Agarwal *et al.*, 1998a). A variety of stimuli that trigger p53-dependent cellular responses increase the ability of p53 to bind to DNA and induce the accumulation of the protein, attributable in large part to an increase in its stability (Maltzman and Czyzyk, 1984; Fritsche *et al.*, 1993; Hupp *et al.*, 1995). Responses to p53 vary in different types of cells and depend on the level of p53 expression. For example, thymocytes undergo p53-dependent apoptosis more readily than do fibroblasts (Lowe *et al.*, 1993; Di Leonardo *et al.*, 1994). In some cells, high levels of p53 induce apoptosis, whereas lower levels induce cell cycle arrest (Chen *et al.*, 1996).

p53 plays an important role in regulating the G2–M transition. Progression from G2 into mitosis was blocked when p53 was overexpressed in either rat or human fibroblasts (Agarwal *et al.*, 1995; Stewart *et al.*, 1995). In addition, the p53-null human fibroblast cell line MDAH041, derived from a Li-Fraumeni patient, continues to enter mitosis when DNA synthesis is blocked by hydroxyurea or inhibited by *N*-

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Abbreviations used: BrdU, 5-bromo-2′deoxyuridine; CAK, cyclin-dependent kinase-activating kinase; CDK, cyclin-dependent kinase; DTT, dithiothreitol; FACS, fluorescence-activated cell sorter; HEK, human embryonic kidney; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPV, human papillomavirus; PCC, premature chromatin condensation.

(phosphonacetyl)-L-aspartate, an inhibitor of de novo pyrimidine nucleotide biosynthesis. Normal regulation is restored when p53 is reexpressed in these cells (Taylor *et al.*, 1999). Although it is clear that p53 is an important regulator of the entry into mitosis, the mechanisms have not been determined.

Entry into mitosis is controlled by the evolutionarily conserved kinase CDC2 (Nurse, 1990), which is regulated by the cell cycle–dependent synthesis and degradation of its activator cyclin B1, which accumulates during G2–M and disappears at the end of mitosis. CDC2 is also regulated by phosphorylation at three sites (Pines, 1995). Phosphorylation of threonine 161 by CDK-activating kinase (CAK) is required for activity (Fisher and Morgan, 1994), whereas phosphorylation of tyrosine 15 by WEE1 (Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993; Watanabe *et al.*, 1995) and threonine 14 by MYT1 (Booher *et al.*, 1997; Liu *et al.*, 1997) inhibits CDC2 activity, keeping the CDC2/cyclin B1 complex inactive during G2. At the onset of mitosis, the phosphatase CDC25C dephosphorylates tyrosine 15 and threonine 14, activating CDC2 (Draetta and Eckstein, 1997). Proteins of the 14-3-3 family bind to CDC25C, blocking its ability to activate CDC2 (Peng *et al.*, 1997). Binding to 14-3-3 proteins is stimulated by phosphorylation of CDC25C at serine 216 by the kinase C-TAK1 during the G1, S, and G2 phases of the cell cycle (Peng *et al.*, 1997, 1998). At the G2–M transition, an unknown signal causes the dephosphorylation of serine 216, triggering the release of 14-3-3, CDC25C-dependent activation of CDC2, and entry into mitosis. DNA damage activates CHK1 and CHK2 (Furnari *et al.*, 1997; Sanchez *et al.*, 1997; Matsuoka *et al.*, 1998), both capable of phosphorylating serine 216 of CDC25C, which may help to explain the arrest of human cells in G2 in response to DNA damage (reviewed in Hwang and Muschel, 1998). We find that p53-dependent G2 arrest involves repression of the transcription of *cdc2* and *cyclin B1* and probably also alterations of the intracellular transport of cyclin B1.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Cells were grown in DMEM (Life Technologies, Gaithersburg, MD), supplemented with antibiotics and 10% fetal bovine serum (Life Technologies), in a humidified atmosphere containing 10% CO_2. MDAH041, a spontaneously immortalized Li-Fraumeni skin fibroblast cell line, was obtained from M. Tainsky (M. D. Anderson Cancer Center, Houston, TX) (Yin *et al.*, 1992). The TR9-7 cell line, expressing a tetracycline-regulated p53 cDNA, was derived from MDAH041 cells (Agarwal *et al.*, 1995). Mimosine (Sigma, St. Louis, MO) was used at a concentration of 200 μM. Cell cycle distribution was determined by fluorescence-activated cell sorter (FACS) analysis of propidium iodide–stained nuclei, and the data were analyzed using CELLFIT software (Becton Dickinson, Franklin Lakes, NJ). Twenty thousand cells were analyzed in each experiment. Recombinant adenoviruses, provided by David Morgan (University of California, San Francisco, CA) (Jin *et al.*, 1998), were amplified by infection of human embryonic kidney (HEK)293 cells (American Type Culture Collection, Manassas, VA) followed by collection of the cell supernatant fluids 48 h later. Debris was removed with a 0.2 μm filter. Viral titers were determined by infecting confluent monolayers of HEK293 cells for 2.5 h, removing the medium that contains the virus, and overlaying the cells with a solution of 1% low-melting agarose (FMC, Rockland, ME) in DMEM plus 10% fetal bovine serum. Plaques were counted 1 week later. All recombinant adenoviruses used lack the E1 and E3 genes. Because the control viruses (for example, tTa) did not induce mitosis, the possibility that other viral products, such as E4, might be expressed does not affect the conclusions drawn from these experiments (see Figure 11 and accompanying text).

Time-Lapse Video Microscopy

Time-lapse analysis was performed as described previously (Taylor *et al.*, 1999). Briefly, NIH Image software was used to capture a series of frames every 16 min 40 s to generate a movie. Video images were captured with a charge-coupled device camera and a Macintosh computer. Cells on coverslips were maintained on an inverted microscope with a heated stage in the presence of 5% CO_2. Mitotic cells entered metaphase appearing rounded and refractile, and cell division followed.

Analysis of DNA Synthesis

5-Bromo-2′-deoxyuridine (BrdU; Sigma) was added to cells for 1.5 h. The cells were fixed in 70% ice-cold ethanol, incubated in 0.08% pepsin (Sigma) in 0.1 M HCl for 20 min at 37°C, collected by centrifugation, incubated in 2 M HCl for 20 min at 37°C and in 0.1 M sodium borate for 10 s, collected by centrifugation, incubated with anti-BrdU-FITC (Becton Dickinson, San Jose, CA) for 30 min, and incubated with propidium iodide (50 μg/ml) and RNAse A (5 μg/ml) for 15 min before FACS analysis (White *et al.*, 1990; Kastan *et al.*, 1991; Taylor *et al.*, 1999).

Chromosome Condensation Assay

Cells were trypsinized, incubated in 75 mM KCl for 15 min at 37°C, fixed in fresh methanol:acetic acid (3:1, vol/vol) for 20 min on ice, and dropped from a distance of 1 m onto glass slides (Smith *et al.*, 1990). The slides were dried in air, and the DNA was stained with 5 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) for 10 min at room temperature. To test the effect of recombinant adenoviruses on the condensation of chromatin, infected cells (including floating cells) were collected by trypsinization and analyzed as described above. These experiments were performed with $\sim 1 \times 10^6$ cells per 10-cm plate and varying amounts of virus. Premature chromatin condensation (PCC) was characterized by condensation of chromatin without formation of distinct chromosomes (see Figure 11). In approximately one-half of the cells with PCC, the nuclear envelope disassembled or broke during preparation; in the others, the condensation of chromatin was evident in intact nuclei without disassembly of the nuclear envelope. Both of these events were scored as PCC. Normal chromatin condensation was defined as formation of normal mitotic chromosomes (see Figure 11).

Western Analysis

Extracts were prepared by lysing cells in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, 2 μg/ml aprotinin, 25 μg/ml leupeptin, 5 μg/ml pepstatin A, and 1 mM dithiothreitol (DTT). Extracts containing equal quantities of proteins, determined by the Bradford method (Bio-Rad, Hercules, CA), were separated by SDS-PAGE (12.5% acrylamide) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were probed with monoclonal antibodies specific for p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA), CDC2 (17; Santa Cruz Biotechnology), and cyclin B1 (GNS1; Santa Cruz Biotechnology) and rabbit polyclonal antibodies specific for CDK7 (C-19; Santa Cruz Biotechnology), cyclin H (C-18; Santa Cruz Biotechnology), CDC2 (C-19; Santa Cruz Biotechnology), WEE1 (wee1-x; Upstate Biotechnology, Lake Placid, NY), CDC25C (C-20; Santa Cruz Biotechnology), 14-3-3β (K-19; Santa Cruz Biotechnology; pan reactive), and 14-3-3σ (a gift from Julio Celis, Århus University, Århus, Denmark). Bound antibodies

were detected with goat anti-mouse or goat anti-rabbit antibody conjugated to horseradish peroxidase (Hoffman La Roche, Basel, Switzerland), using enhanced chemiluminescence (Dupont, Wilmington, DE) (Agarwal *et al.*, 1995). NIH Image software was used to quantitate digital images from scanned autorads.

Measurement of CDC2 Kinase and CAK Activities

Cell lysates prepared with 50 mM HEPES, pH 7.0, 250 mM NaCl, 0.1% nonidet P-40, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, 2 μg/ml aprotinin, 25 μg/ml leupeptin, 5 μg/ml pepstatin A, and 1 mM DTT, containing equal quantities of protein, were incubated with either a CDC2-specific monoclonal antibody (17; Santa Cruz Biotechnology), a polyclonal antibody raised against the C terminus of CDC2 (Babco, Richmond, CA) (Rosenblatt *et al.*, 1992), or a monoclonal antibody specific for cyclin B1 (GNS1; Santa Cruz Biotechnology). Immune complexes were isolated with protein A–conjugated sepharose (Pharmacia, Bridgewater, NJ). Sepharose pellets were washed with lysis buffer and incubated for 30 min at 37°C in 20 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 1 μg of histone H1 (Hoffman La Roche), 1 mM DTT, 100 μM unlabeled ATP, and 10 μCi of [γ-^{32}P]ATP in a total volume of 20 μl. Phosphorylated histone was separated by SDS-PAGE (12.5% acrylamide), and the dried gels were exposed to film. Phosphorylation was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Cell lysates prepared as described for the CDC2 kinase assay were immunoprecipitated with an antibody specific for cyclin H (C-18; Santa Cruz Biotechnology), a subunit of CAK, and the immune complexes were collected with protein A–conjugated sepharose and then incubated for 30 min at 37°C with 2 μg of purified CDK2 glutathione *S*-transferase fusion protein (Tsai *et al.*, 1991; Hitomi *et al.*, 1998), ^{32}P-labeled ATP, and all additional kinase buffer components described for the CDC2 kinase assay except histone H1. Phosphorylated CDK2 was separated by SDS-PAGE (12.5% acrylamide), the dried gels were exposed to film, and phosphorylation was quantitated with a PhosphorImager.

Other Assays

To analyze tyrosine phosphorylation, CDC2 was immunoprecipitated from cells lysed as described for Western analysis by the use of a monoclonal antibody and protein-A sepharose, separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with an antibody specific for phosphotyrosine (PY99; Santa Cruz Biotechnology), conjugated directly to horseradish peroxidase. Antibody binding was detected using enhanced chemiluminescence (Dupont) (Agarwal *et al.*, 1995). Total CDC2 levels were confirmed by probing the same membrane with a polyclonal antiserum to CDC2.

Cyclin B1 and associated proteins were immunoprecipitated as described for measurement of CDC2 kinase activity, using a monoclonal antibody to cyclin B1. Western transfers were then probed with a polyclonal antibody to p21/waf1 directly conjugated to horseradish peroxidase (C-19: Santa Cruz Biotechnology) and were detected by enhanced chemiluminescence.

For Northern analysis, total RNA was extracted with Trizol reagent (Life Technologies), according to the manufacturer's instructions, separated by electrophoresis in a denaturing agarose gel, transferred to Hybond-N+ nylon membranes (Amersham, Arlington Heights, IL), and probed with ^{32}P-labeled DNA probes for *cdc2* or *cyclin B1* (Sambrook *et al.*, 1989).

cdc2 and *cyclin B1* promoter activities were determined by measuring luciferase activity in pools of TR9-7 cells stably transfected with constructs containing either 3200, 245, 128, 114, 104, or 74 bp of *cdc2* upstream promoter sequence driving the expression of luciferase (Sugarman *et al.*, 1995) or with constructs containing either 555, 287, or 123 bp of the *cyclin B1* promoter upstream of luciferase (Katula *et al.*, 1997). Luciferase activity was corrected for loading by comparing the total protein concentrations in each lysate, determined by the Bradford method (Bio-Rad).

RESULTS

Effect of p53 on CAK and the CDC2/Cyclin B1 Complex

We investigated the mechanism of G2 arrest by p53 in TR9-7 cells, which contain a tetracycline-regulated wild-type p53 gene in the p53-null MDAH041 Li-Fraumeni human fibroblast cell line (Agarwal *et al.*, 1995). TR9-7 cells were released from a mimosine block, which arrests them reversibly at the beginning of S phase (Hughes and Cook, 1996), and deprived of tetracycline to induce p53-mediated arrest, mainly in G2 (Agarwal *et al.*, 1995). G2 arrest was evident 48 h after removal of mimosine in the absence of tetracycline, whereas cells incubated in the presence of tetracycline have progressed through G2 and M by this time (Agarwal *et al.*, 1995). We measured BrdU incorporation to define the kinetics of DNA synthesis after removal of mimosine and to determine when the cells first encounter the G2 block. Cells incorporated BrdU 4 h after removal of mimosine, either in the presence or absence of tetracycline (Figure 1A). The percentage of cells incorporating BrdU decreased at 19 h, indicating that DNA synthesis was nearing completion (Figure 1, A and B). We analyzed entry into mitosis by the use of time-lapse video microscopy in cells released from a mimosine block in the presence of tetracycline. Cells started to enter mitosis ~20 h after mimosine removal (Figure 1C), consistent with the timing of completion of DNA synthesis (Figure 1, A and B). In the absence of tetracycline, some cells (~5%) entered mitosis between 20 and 25 h after release from a mimosine block, after which entry into mitosis was blocked (Figure 1C). Cells that could enter mitosis up to 25 h had probably been blocked by mimosine in late S phase. Upon the removal of mimosine, these cells would have completed DNA synthesis and progressed through G2 before p53 had accumulated to a high enough concentration to block entry into mitosis. Importantly, time-lapse analysis showed that many TR9-7 cells released from the mimosine block in the absence of tetracycline did not enter mitosis but maintained an interphase morphology with an intact nucleus (our unpublished data), suggesting that the point of arrest is in G2 or very early in M, before chromosome condensation and disassembly of the nuclear membrane.

We prepared duplicate plates of cells to analyze cell cycle distribution, protein levels, and kinase activity. Twenty-two hours after mimosine was removed, many cells incubated with or without tetracycline contained a 4N content of DNA (Figure 2A), showing that most had completed DNA synthesis but not mitosis. CDC2 kinase activity, determined by the phosphorylation of histone H1 by immune complexes, formed with an antibody either to cyclin B1 or to the C terminus of CDC2, was reduced by 65 and 69%, respectively, when p53 was induced (Figure 2, B and C). Therefore, CDC2 activity is reduced in response to p53 before the majority of cells have entered mitosis.

CAK, which activates CDC2, is inhibited in response to ionizing radiation in normal but not p53-null mouse embryo fibroblasts and can also be inhibited by purified p53 in vitro (Schneider *et al.*, 1998). CAK, composed of CDK7, cyclin H, and MAT1 (Yankulov and Bentley, 1997), was assayed by

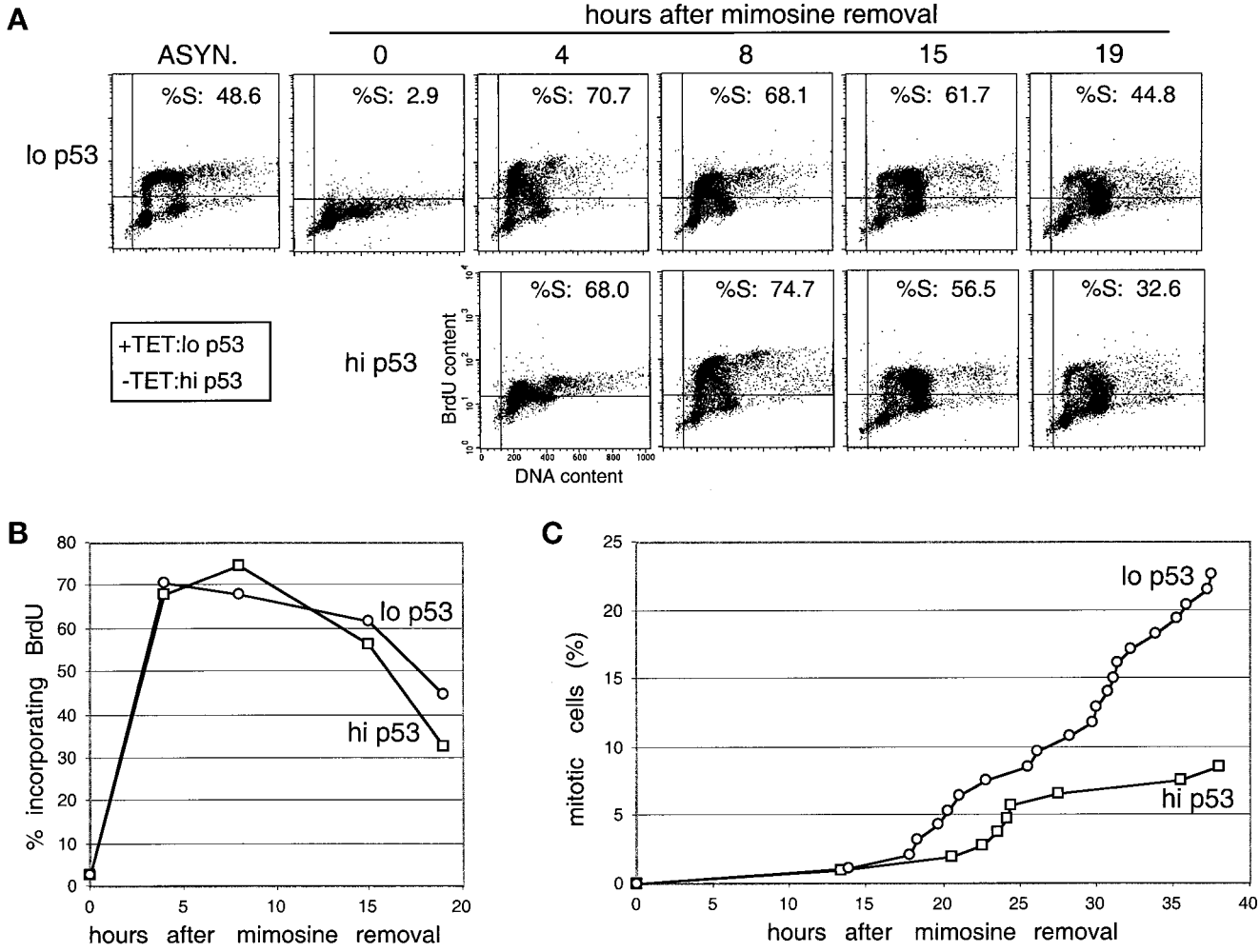

Figure 1. Time courses of DNA synthesis and mitosis after release from a mimosine block. TR9-7 cells were incubated for 48 h in the presence of 200 μM mimosine and tetracycline (TET). Mimosine was removed, and the cells were analyzed for BrdU incorporation or entry into mitosis. (A) FACS analysis of BrdU incorporation. At the time mimosine was removed, tetracycline was either removed (high [hi] p53) or retained (low [lo] p53). The cells were pulsed with BrdU for 1.5 h at various times and analyzed by FACS by the use of a fluorescently labeled antibody to BrdU. ASYN. refers to TR9-7 cells growing asynchronously in the presence of tetracycline. The 0 h time point refers to cells pulsed with BrdU in the presence of mimosine (lo p53); %S refers to the percentage of cells incorporating BrdU (top right quadrant). (B) Graphical presentation of %S in A. (C) Resumption of mitosis after release of TR9-7 cells from a mimosine block. Mitosis was analyzed by time-lapse video microscopy of a field of 100 cells. Filming was begun when mimosine and tetracycline were removed.

immunoprecipitating the complex with an antibody to cyclin H and incubating it with ^{32}P-labeled ATP and CDK2, a good substrate. p53 status had no effect on CAK activity 22 h after release from a mimosine block (Figure 2D), and thus loss of CDC2 activity in response to p53 is not caused by inactivation of CAK. Inhibitory tyrosine phosphorylation was analyzed by immunoprecipitating CDC2, followed by Western analysis with an antibody to phosphotyrosine. Tyrosine phosphorylation was reduced in cells arrested in G2 in response to p53 (Figure 2E). When the same membrane was reprobed with an antibody to CDC2, we observed two major species of CDC2 (Figure 2E). Phosphorylation of CDC2 at threonine 14 and tyrosine 15 decreases its electrophoretic mobility (Poon *et al.*, 1997). The top band in Figure 2E could be resolved into two bands upon more extensive electrophoresis (our unpublished data). Therefore, this band probably represents a mixture of CDC2 phosphorylated at threonine 14, tyrosine 15, or both (Poon *et al.*, 1997). The reduction in intensity of this band is consistent with the observed reduction in tyrosine phosphorylation (Figure 2E). The amount of CDC2 protein was unchanged after 22 h (Figure 2E), but cyclin B1 levels were reduced by 40% in cells arrested by p53 (Figure 2C). Most uninduced control cells had not progressed through mitosis at this time (Figure 2A), showing that the downregulation of cyclin B1 occurs soon enough to affect the G2–M transition.

p53 overexpression induced an efficient G2 arrest in cells analyzed 48 or 72 h after release from a mimosine block (Figure 3A). After 48 h in the presence of p53 there was a marked reduction in the amount of CDC2 and an

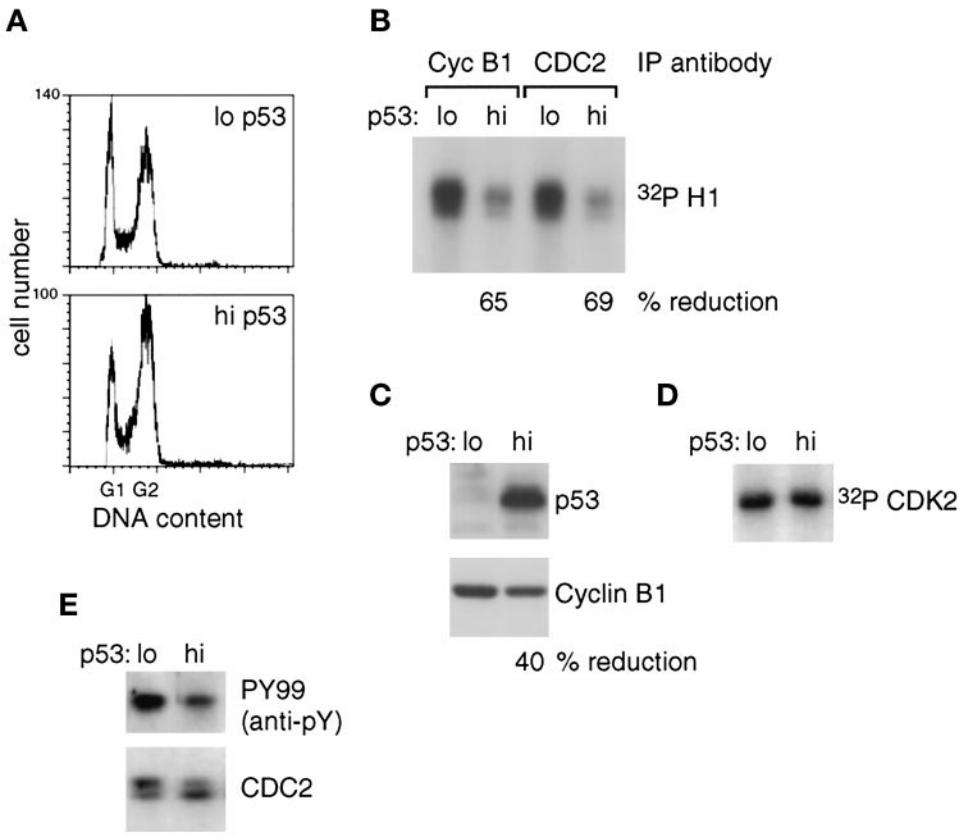

Figure 2. Cell cycle distribution and analysis of CDC2/cyclin B1, p53, and CAK in TR9-7 cells 22 h after the removal of mimosine. Cells synchronized in S phase by treatment with mimosine for 48 h were incubated for 22 h in the presence (lo p53) or absence (hi p53) of tetracycline. (A) Cell cycle distribution. DNA content was determined by FACS analysis. (B) CDC2 activity. Histone H1 was phosphorylated in vitro by the use of [γ-^{32}P]ATP, and immunoprecipitates (IPs) were prepared either with a monoclonal antibody specific for cyclin B1 (Cyc B1) or with a polyclonal antiserum specific for the C terminus of CDC2. The products were analyzed by SDS-PAGE and autoradiography. The amount of radioactive phosphate incorporated into histone H1 when p53 was induced was determined with a PhosphorImager and compared with the amount in cells without p53 induction (% reduction). (C) Expression of p53 and cyclin B1. Western analyses are shown. The levels of cyclin B1, determined as described in MATERIALS AND METHODS, were compared in the absence and presence of tetracycline (% reduction). (D) CAK activity. Activity was determined by phosphorylation of recombinant CDK2 by cyclin H immunoprecipitates in the presence of [γ-^{32}P]ATP. (E) Expression and phosphorylation of CDC2. Tyrosine phosphorylation of CDC2 was determined after immunoprecipitation and Western transfer by the use of anti-phosphotyrosine (PY99). Total CDC2 was detected with anti-CDC2.

almost complete loss of cyclin B1 (Figure 3B). After 72 h, both proteins were essentially absent. Fewer cells with low p53 had a 4N DNA content 72 h after mimosine removal than after 48 h (Figure 3A), suggesting that some of the synchronized cells were still progressing through mitosis between 48 and 72 h. Therefore, CDC2 was downregulated by p53 soon enough to block the entry of some of the synchronized cells into mitosis. Not surprisingly, the kinase activity of CDC2 was greatly reduced in cells arrested for 48 or 72 h, compared with control cells or cells arrested with nocodazole, which blocks the polymerization of tubulin and traps cells in mitosis because the spindle cannot form (Figure 3C).

Time-lapse analysis revealed that, when p53 was induced, cells were blocked from entering mitosis, placing the arrest point somewhere between G2 and the beginning of M. To confirm this conclusion, we used nocodazole to trap cells in mitosis. If cells exposed to high levels of p53 had attempted mitosis, CDC2 kinase activity would not be suppressed in the presence of nocodazole (Draetta and Beach, 1988). The cells were simultaneously deprived of tetracycline and released from a mimosine block, and nocodazole was added just before control cells would have started to enter mitosis (18 h; Figure 1C). Ten hours later, cells incubated with or without induction of p53 contained mainly a 4N content of DNA, showing that most cells had completed DNA synthesis (Figure 4A). However, CDC2 kinase activity was reduced by 61% in the cells exposed to high p53 (Figure 4B), suggesting that they have arrested in G2 before attempting mitosis. The level of cyclin B1 was reduced by 45% in cells expressing a high level of p53 28 h after release from a mimosine block in the presence of nocodazole (Figure 4C). CAK activity was not affected by the induction of p53 (Figure 4D), tyrosine phosphorylation of CDC2 was reduced slightly, and the level of total CDC2 protein was essentially unchanged (Figure 4E). There was a slight reduction in the abundance of the most slowly migrating species of CDC2 (phosphorylated on both threonine 14 and tyrosine 15; compare with data of Poon *et al.* [1997]) in the presence of a high level of p53, consistent with the reduction in phosphotyrosine detected with an anti-phosphotyrosine antibody (Figure 4E). Therefore, p53 blocks cells in G2. We also noticed that, after mimosine was removed and p53 was induced, some cells retained a 2N content of DNA (Figure 4A). Because all cells were prevented from completing mitosis with nocodazole, the 2N cells probably were unable to resume DNA synthesis under these conditions.

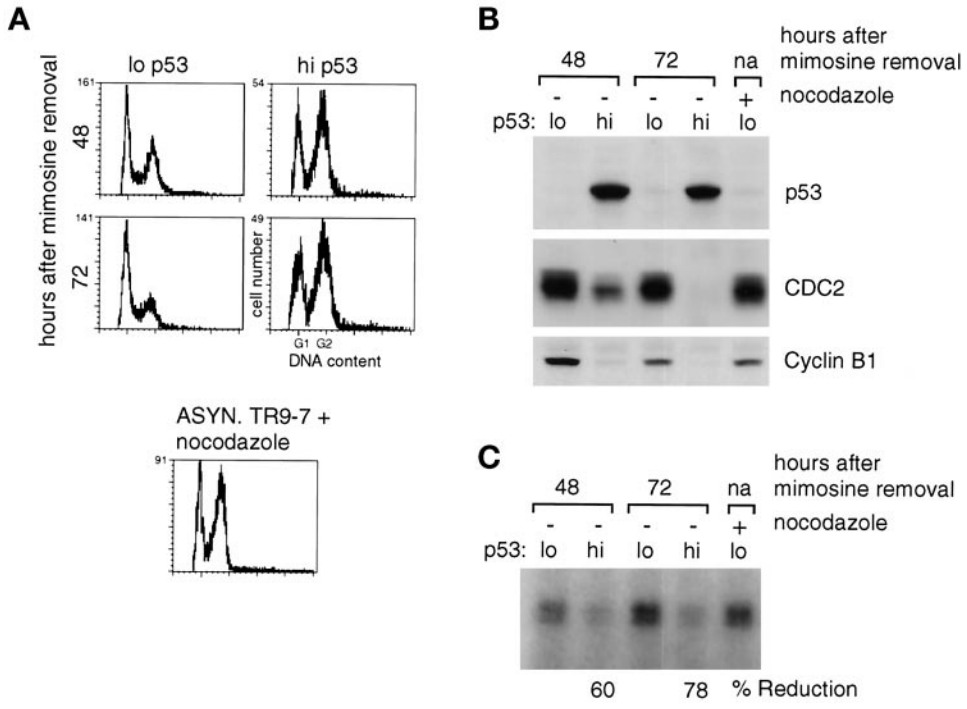

Figure 3. Cell cycle distribution, expression, and activity of CDC2/cyclin B1 in TR9-7 cells after the removal of mimosine. Cells were released from a mimosine block in the presence (lo p53) or absence (hi p53) of tetracycline and analyzed 48 or 72 h later. (A) Cell cycle distribution of TR9-7 cells 48 or 72 h after mimosine removal. For comparison, cells arrested in mitosis were obtained by incubating asynchronously (ASYN) growing TR9-7 cells in nocodazole for 16 h. (B) Expression of p53, CDC2, and cyclin B1, detected by Western transfer. (C) CDC2 activity. The ability of CDC2 immunoprecipitated with a monoclonal antibody to phosphorylate histone H1 was determined; % reduction was determined as described in Figure 2B. na, not applicable.

Effect of p53 on Transcription of the cdc2 and *cyclin B1 Genes*

The levels of CDC2 and cyclin B1 proteins might be decreased in any of several ways. Because ionizing radiation leads to a decrease of *cdc2* and *cyclin B1* mRNA in normal but not p53-null cells (Azzam *et al.*, 1997; de Toledo *et al.*, 1998), we measured *cdc2* and *cyclin B1* mRNA levels in cells released from a mimosine block and arrested in G2 because of overexpression of p53. p53 suppressed *cdc2* and *cyclin B1* mRNA expression at 24, 48, and 72 h after induction (Figure 5A). FACS analysis of cells from duplicate plates analyzed 72 h after removal of mimosine confirmed that many cells were arrested in G2 (Figure 5B). Therefore, overexpression of p53 downregulates both *cdc2* and *cyclin B1* at the mRNA level in cells arrested in G2, and the protein levels are downregulated with similar kinetics.

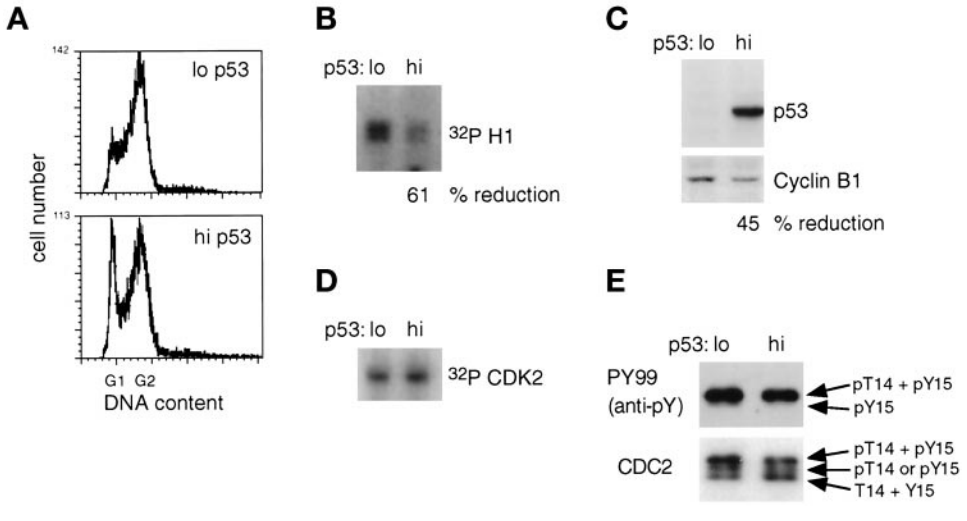

Figure 4. Cell cycle distribution, cyclin B1 expression, and CDC2 activity in TR9-7 cells 28 h after the removal of mimosine in the presence of nocodazole. TR9-7 cells were released from a mimosine block in the presence (lo p53) or absence (hi p53) of tetracycline, and nocodazole was added 18 h later. Samples were analyzed after an additional 10 h. (A) Cell cycle distribution of TR9-7 cells after mimosine was removed and nocodazole was added. (B) CDC2 activity, assessed as described in Figure 3. (C) Expression of p53 and cyclin B1. The proteins were detected by Western transfer, and % reduction is shown. (D) CAK activity, assessed as described in Figure 2. (E) Tyrosine phosphorylation of CDC2 in TR9-7 cells 28 h after mimosine was removed. Phosphorylation of CDC2 on tyrosine was determined as described in Figure 2. pT, phosphothreonine; pY, phosphotyrosine.

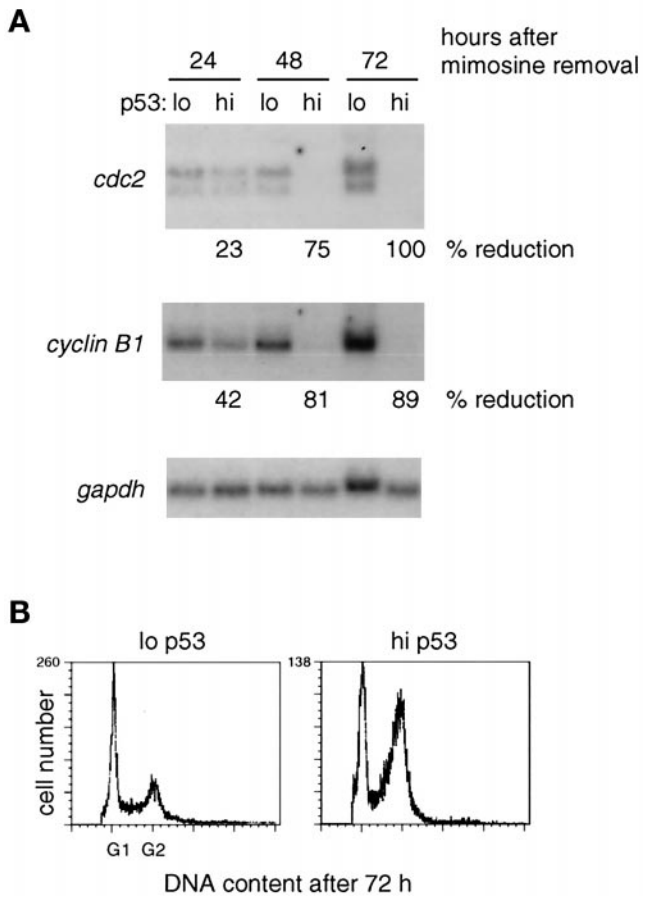

Figure 5. *cdc2* and *cyclin B1* mRNA levels in cells arrested in G2 because of overexpression of p53. A Northern transfer was used to detect *cdc2* and *cyclin B1* mRNAs in TR9-7 cells after release from a mimosine block in the presence (lo p53) or absence (hi p53) of tetracycline. (A) *cdc2* and *cyclin B1* mRNAs. Northern transfers were probed for *cdc2*, *cyclin B1,* and *gapdh* (as a loading control); % reductions were calculated by the use of a PhosphorImager to measure signal intensities, which were also corrected for loading. (B) Cell cycle analysis. Duplicate plates were used to prepare RNA for the analysis shown in A and for FACS analysis to determine cell cycle distributions 72 h after removal of mimosine.

To test for transcriptional repression, we transfected TR9-7 cells with a luciferase reporter gene driven by 3200 bp of *cdc2* upstream promoter sequences (Sugarman *et al.*, 1995). To approximate as closely as possible the regulation of the chromosomal *cdc2* gene, we isolated pools of clones in which the *cdc2* reporter construct was stably integrated into genomic DNA. *cdc2* promoter activity was repressed 4 h after removing tetracycline from asynchronously growing TR9-7 cells and was reduced by 88% 48 h after tetracycline removal (Figure 6A). Under these conditions 70% of the cells arrest in G1, with 7% in S phase and 23% in G2 and/or M (Agarwal *et al.*, 1995). The effect of p53 on the *cdc2* promoter might be explained by the ability of p53 to arrest cells in G1, because transcription of *cdc2* is cell cycle dependent, with the lowest rate during G1 and much higher rates in S and G2 (Welch and Wang, 1992). Therefore, we measured *cdc2* promoter activity after the pool of TR9-7 cells containing the *cdc2* luciferase construct had been arrested in G2 by the use of the mimosine synchronization protocol. To obtain more cells arrested in G2, we preinduced p53 by removing tetracycline 48 h after adding mimosine and incubating the cells for 16 h more in the continued presence of mimosine, to allow high levels of p53 to accumulate before release into S phase. Luciferase activity was repressed by 69% 72 h after p53 induction, when most of the cells were arrested in G2, showing that the repression of *cdc2* transcription by p53 is not caused by synchronization of the cells in G1 (Figure 6B).

To define the region of the *cdc2* promoter required for repression by p53, we isolated pools of TR9-7 cells transfected stably with constructs containing progressive deletions from the 5′ end of the *cdc2* promoter, linked to luciferase. The cells were released from a mimosine block to arrest them in G2 (Figures 2 and 3). Promoter fragments containing sequences up to −104 were repressed by p53 (Figure 6C). Further deletion to −74 caused an ~20-fold drop in promoter activity and eliminated repression by p53 (Figure 6C). This region contains a CAAT box shown previously to be important for transcription (Figure 6D) (Sugarman *et al.*, 1995). Therefore, sequences between −104 and −74 are required for repression of the *cdc2* promoter by p53, and the basal activity of the promoter is not adversely affected by p53.

To determine whether p53 could also repress the *cyclin B1* promoter, we isolated a pool of TR9-7 cells stably transfected with a construct containing 555 bp of this promoter upstream of a luciferase reporter gene (Nuckolls *et al.*, 1998). This region of the promoter is sufficient for normal regulation of transcription during the cell cycle (Hwang *et al.*, 1995). Cells were synchronized by treating them with mimosine for 48 h, followed by preinduction of p53 for 16 h as described above and analysis at two times after mimosine removal. Luciferase activity was reduced by 35 or 55% at 24 or 32 h after mimosine removal, respectively, when most of the cells were arrested in G2 (Figure 7A). Therefore, p53 can repress the *cyclin B1* promoter in cells arrested in G2. Constructs containing various regions of the *cyclin B1* promoter were used to map the region required for repression by p53. Pools of TR9-7 cells stably transfected with *cyclin B1* deletion constructs were arrested in G2. Sequences to −287 were sufficient for repression by p53, whereas deletion to −123 reduced repression (Figure 7B). The intervening region contains several protein-binding elements that are potential targets of repression (Figure 7C).

Downregulation of CDC2 Expression by p53

In cells arrested in G2 by p53 overexpression, the levels of cyclin B1 decreased first, followed by a decrease in CDC2 (Figures 2–4). We analyzed the effect of p53 induction on several cell cycle proteins in asynchronously growing TR9-7 cells. CDC2 protein levels began to drop 24 h after p53 was induced, and by 36 h, very little CDC2 remained (Figure 8). p53 induction started at 8 h and was maximal by 16 h. Thus, there was a lag of at least 8 h between maximal p53 induction and the initiation of CDC2 downregulation. The levels of CDC25C were also reduced 36 h after p53 induction. However, the levels of WEE1, CDK7, and cyclin H were relatively unaffected by p53 overexpression, suggesting that the effect of p53 on CDC2 and cyclin B1 is not caused by a

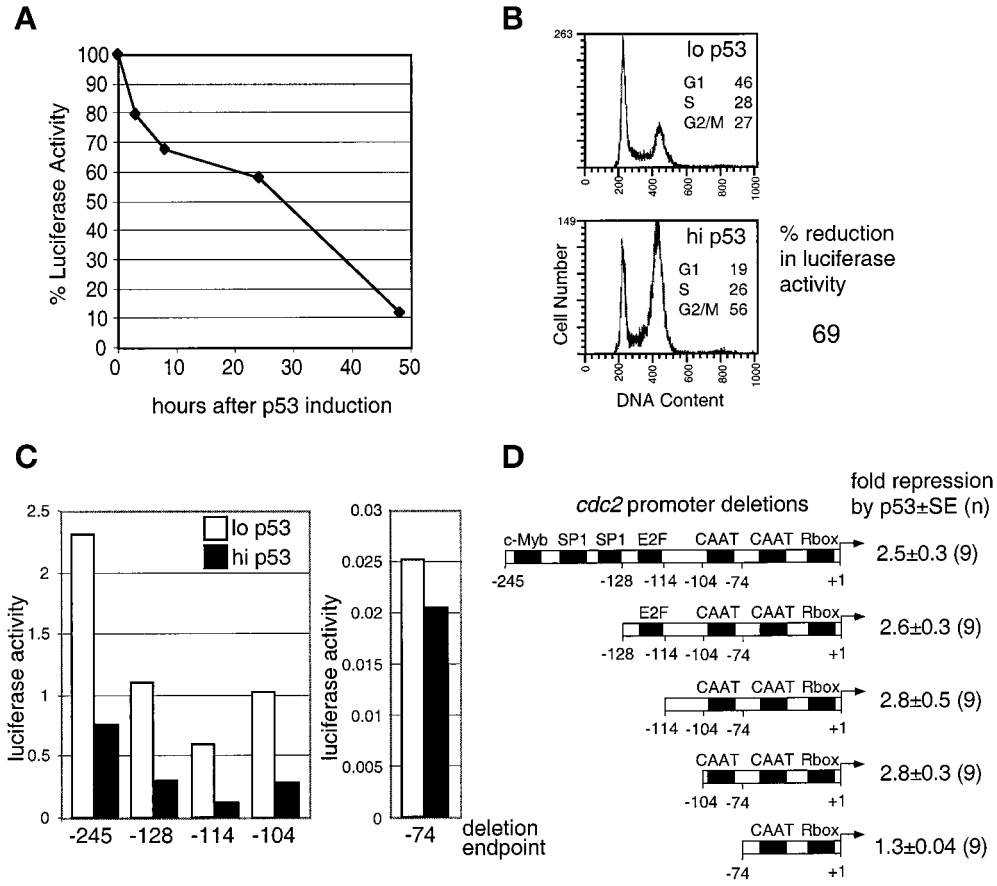

Figure 6. Effect of p53 overexpression on *cdc2* promoter activity. TR9-7 cells were transfected with *cdc2* promoter–luciferase constructs. Pools of clones containing stably integrated reporter constructs were tested for repression by p53. The pools were incubated without tetracycline to induce p53, and the luciferase activity was measured and corrected for protein concentration (MATERIALS AND METHODS). (A) *cdc2* promoter activity after removal of tetracycline from asynchronous cultures. Luciferase activity is shown relative to the activity at the beginning of the experiment. (B) Cell cycle distribution and *cdc2* promoter activity in cells arrested in G2 by p53. Cells were incubated for 48 h in mimosine and then for 16 h in the presence (lo p53) or absence (hi p53) of tetracycline in the continued presence of mimosine. Mimosine was removed, cells were incubated for 72 h in the presence or absence of tetracycline, and the cells were analyzed for cell cycle position and luciferase activity; % reduction is the level of luciferase activity in the absence of tetracycline relative to the level in the presence of tetracycline. (C) Deletion mapping of the *cdc2* promoter. Deletion constructs containing various lengths of the promoter linked to luciferase were transfected into TR9-7 cells. Pools of stable clones were arrested with mimosine for 48 h and incubated in the absence of mimosine in the presence or absence of tetracycline for 72 h. Luciferase activity, corrected for protein concentrations of cell lysates in a typical experiment, is shown. (D) Diagram of the *cdc2* promoter. Known and predicted DNA-binding elements are shown (Sugarman *et al.*, 1995). The fold repression and SEs from the indicated number (n) of replicate samples are shown. Similar patterns of repression were observed in at least three independent experiments.

general downregulation of cell cycle regulatory proteins. In cells arrested in G2 by p53 induction 30 h after release from a mimosine block, WEE1 was unchanged, and CDC25C was downregulated (our unpublished data).

Effect of p53 Overexpression on p21/waf1 and 14-3-3 Proteins

p53 can transactivate the promoter of the *14-3-3σ* gene (Hermeking *et al.*, 1997). Proteins of the 14-3-3 family can bind to and inhibit CDC25, suggesting that 14-3-3σ induced in response to p53 might cause tyrosine-phosphorylated, inactive CDC2 to accumulate (Peng *et al.*, 1997). We show that, although the induction of p53 in TR9-7 cells causes loss of CDC2 activity, the tyrosine phosphorylation of CDC2 is decreased, suggesting that 14-3-3σ might not be responsible. To address this issue, we analyzed the expression of 14-3-3σ with a subtype-specific antiserum (Leffers *et al.*, 1993). The epithelial tumor cell line HCT116, containing wild-type p53, showed induction of both p53 and 14-3-3σ after treatment with hydroxyurea (Figure 9A). In contrast, 14-3-3σ could not be detected in HT1080 cells, a fibroblast tumor cell line containing wild-type p53 (Figure 9A), and similar results were obtained when HCT116 and HT1080 cells were treated with adriamycin (our unpublished data). A smaller protein was detected faintly in HT1080 and HCT116 cells and was induced slightly by treatment with hydroxyurea. However, this protein was not detected in CDC25C immunoprecipitates and was not detected at all in some immunoblots of total cell extracts (our unpublished data). 14-3-3σ was not detected in TR9-7 cells 24 or 48 h after p53 induction (Figure 9B) (our unpublished data). Furthermore, using an antiserum against a conserved region in the 14-3-3 family of proteins, we did not detect any protein that could be induced by p53 in TR9-7 cells or upon DNA damage in HT1080 cells caused by camptothecin, a topoisomerase I inhibitor that causes p53 to accumulate (Figure 9C). Therefore, although induced by DNA damage in HCT116 cells, 14-3-3σ was not detected in either TR9-7 or HT1080 fibroblast cells in the presence of high levels of p53. This result may explain why the tyrosine phosphorylation of CDC2 does not increase upon p53 induction in TR9-7 cells.

We also analyzed p21/waf1, another transcriptional target of p53 that can inhibit the G2–M transition (Bunz *et al.*,

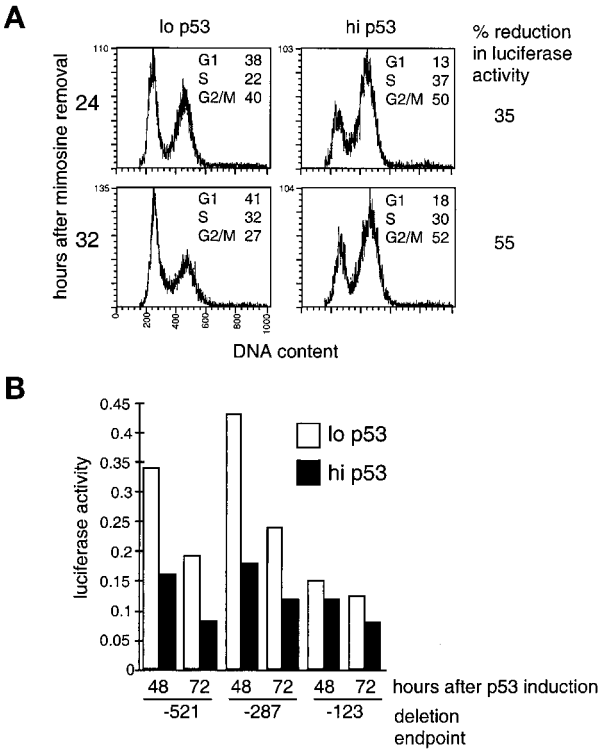

Figure 7. Effect of p53 on *cyclin B1* promoter activity. TR9-7 cells were transfected with promoter–luciferase constructs, and stable pools were released from a mimosine block in the presence (lo p53) or absence (hi p53) of tetracycline. (A) Repression of the *cyclin B1* promoter in cells arrested in G2 by p53. For these experiments, p53 was preinduced in mimosine-arrested cultures as described in Figure 6B. The cell cycle distribution of the TR9-7 pool containing the *cyclin B1* reporter construct 24 or 32 h after mimosine removal is shown. The *cyclin B1* promoter activity in cells arrested in G2 for 24 or 32 h is shown as the % reduction in luciferase activity in the absence of tetracycline compared with the activity from duplicate plates grown in the presence of tetracycline. (B) Deletion mapping of the *cyclin B1* promoter. Constructs containing various regions of the promoter linked to luciferase were transfected stably into TR9-7 cells. Pools of clones were tested for repression by p53. The cells were arrested in G2 as described in A, and luciferase activity, corrected for protein concentration, is shown. (C) Diagram of the *cyclin B1* promoter. Protein-binding elements are shown (Katula *et al.*, 1997). The fold repression and SEs from the indicated number (n) of replicate samples are shown. Similar patterns of repression were observed in at least three independent experiments.

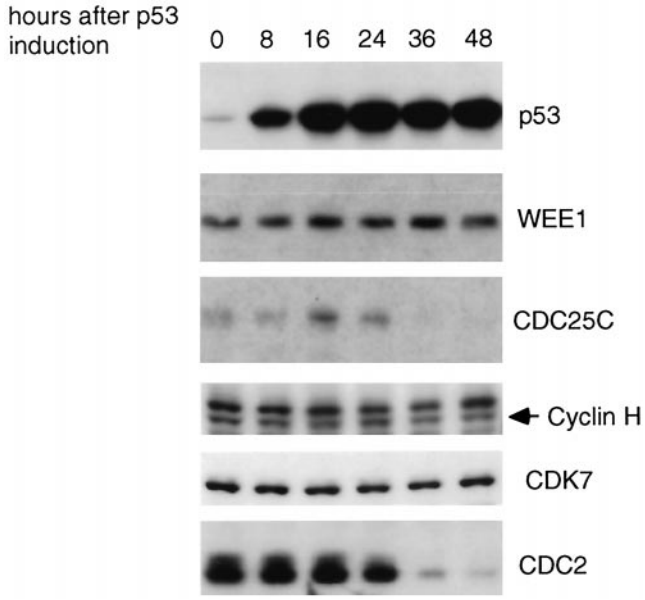

Figure 8. Expression of cell cycle regulatory proteins in response to p53 overexpression. p53 was induced by removing tetracycline from asynchronously growing TR9-7 cells, which were analyzed by Western transfer.

1998; Niculescu *et al.*, 1998). p21/waf1 is expressed highly in TR9-7 cells arrested in G2 by p53 (Agarwal *et al.*, 1995). We immunoprecipitated cyclin B1 and associated proteins from TR9-7 cells 30 h after release from a mimosine block in the presence or absence of tetracycline. p21/waf1 was detected in cyclin B1 immunoprecipitates that, as expected, also contained CDC2 (Figure 10). This result shows that p21/waf1 can associate with cyclin B1 and might contribute to inhibition of the CDC2/cyclin B1 complex.

Targeting of Cyclin B1 to the Nucleus Is Required to Abrogate p53-dependent G2 Arrest

We used recombinant adenoviral vectors to overexpress CDC2 and cyclin B1 (Jin *et al.*, 1998) and a standard chromosome-spreading technique to determine whether any cells had entered mitosis with condensed chromosomes (Smith *et al.*, 1990). TR9-7 cells were released from a mimosine block and incubated for 72 h in the absence of tetracycline to arrest them in G2. The cells were infected and harvested 48 h later. The nuclear-targeted cyclin B1 virus in combination with the CDC2 T14A Y15F virus, but not alone, induced PCC in 15% of the cells (Figure 11, A and B). PCC was characterized by condensed chromatin that did not form distinct mitotic chromosomes (see MATERIALS AND METHODS), indicating that these cells have entered mitosis (Figure 11A). Expression of either the CDC2 T14A Y15F virus or the wild-type cyclin B1 virus alone, or in combination, did not induce PCC (Figure 11B). Similar results were obtained when cells were infected at the time of mimosine removal and analyzed 48 h later (our unpublished data).

We used immunofluorescence with an antibody to a hemagglutinin epitope tag fused to CDC2 expressed from the

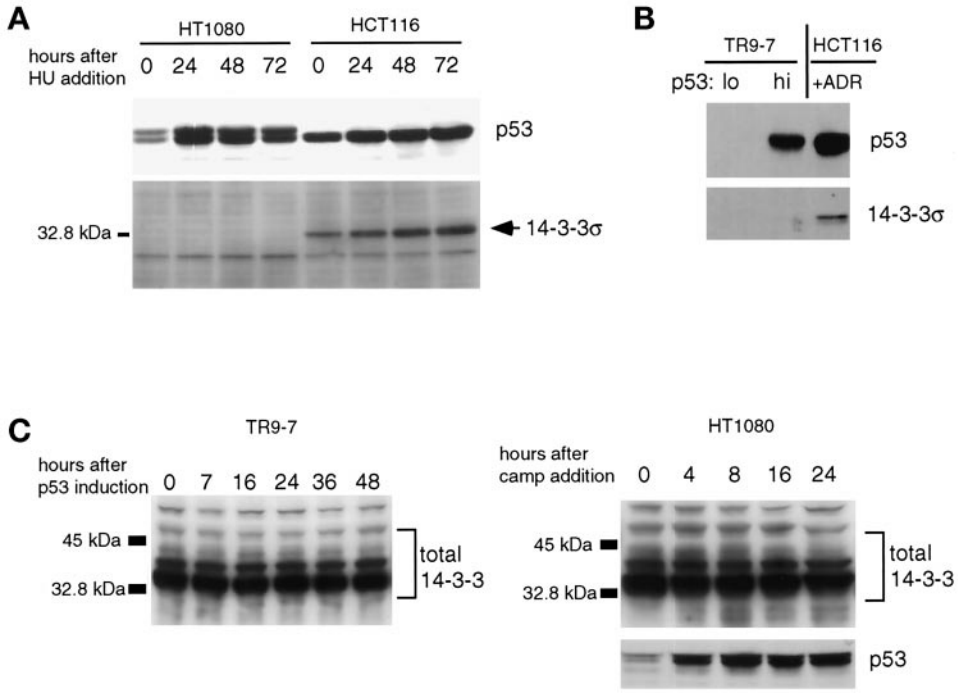

Figure 9. Expression of 14-3-3 proteins in response to the overexpression of p53. p53 was induced by removing tetracycline from asynchronously growing TR9-7 cells or by treating the tumor cell lines HT1080 and HCT116, which have wild-type p53, with adriamycin (ADR; 0.2 μg/ml), hydroxyurea (HU; 2 mM), or camptothecin (camp; 0.5 μg/ml). HT1080 cells are from a fibrosarcoma, and HCT116 cells are of epithelial origin. (A) Western analyses with antisera specific for 14-3-3σ and p53. (B) Expression of 14-3-3σ and p53 in TR9-7 and HCT116 cells. TR9-7 cells were incubated with (lo p53) or without (hi p53) tetracycline for 24 h, and HCT116 cells were treated with ADR for 48 h. (C) Western analyses of TR9-7 and HT1080 cells with a polyclonal antiserum broadly reactive to proteins of the 14-3-3 family and an antibody to p53.

recombinant adenovirus to determine that ~40% of TR9-7 cells were infected in these experiments (our unpublished data). Because not all cells are infected, CDC2 T14A Y15F and nuclear-targeted cyclin B1 are more efficient in inducing mitosis than indicated by the mitotic index of 15%, which also includes uninfected cells. Immunofluorescence with an antibody to cyclin B1 confirmed a previous report (Jin *et al.*, 1998) that the exogenous wild-type cyclin B1 expressed in adenovirus-infected cells (determined by its high level of expression compared with endogenous cyclin B1 in uninfected cells) was localized primarily in the cytoplasm, whereas the nuclear-targeted cyclin B1 was found mainly in the nucleus (our unpublished data). The expression of CDC2 T14A Y15F was high, whereas exogenous wild-type cyclin B1 was expressed less efficiently than was the nuclear-targeted cyclin B1 protein, but all three exogenous proteins were in excess over the endogenous CDC2 and cyclin B1 (Figure 11B). We therefore increased the multiplicity of infection of the wild-type cyclin B1 virus. At the highest level of wild-type cyclin B1 expression, 2.6% of the cells entered mitosis, whereas much lower levels of nuclear cyclin B1 were required to induce mitosis in 8.5% of cells (Figure 11C). Therefore, targeting cyclin B1 to the nucleus is required to overcome the G2 arrest induced by p53.

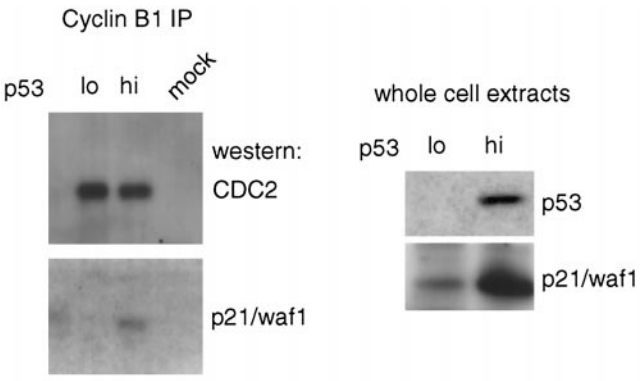

Figure 10. Western analysis of cyclin B1 immunoprecipitates for p21/waf1. TR9-7 cells were released from a mimosine block in the presence (lo p53) or absence (hi p53) of tetracycline. Thirty hours later, cell lysates were analyzed directly or immunoprecipitated with an antibody to cyclin B1. Whole-cell extracts and immunoprecipitates (IPs) were probed with an antibody to p21/waf1 conjugated directly to horseradish peroxidase. Immunoprecipitates were also analyzed with an antibody to CDC2. The lane labeled "mock" corresponds to cyclin B1 antibody that was not incubated with a lysate.

DISCUSSION

The G2–M Transition Is Regulated by p53

By blocking the G1–S transition, p53 ensures that damaged DNA is not replicated and that cells do not synthesize DNA under suboptimal conditions (reviewed in Ko and Prives, 1996; Levine, 1997; Agarwal *et al.*, 1998b). Overexpression of p53 in fibroblasts also causes arrest in G2 (Agarwal *et al.*, 1995; Stewart *et al.*, 1995), and inactivation of p53 by human papillomavirus (HPV)-E6 attenuates the G2 delay that normally occurs in response to ionizing radiation (Thompson *et al.*, 1997). However, the ability of HPV-E6 to bind to other cellular proteins (Chen *et al.*, 1995; Imai *et al.*, 1997; Kiyono *et al.*, 1997; Gao *et al.*, 1999) limits the interpretation of these findings. A truncated form of p53 that can inhibit endogenous p53 blocked DNA

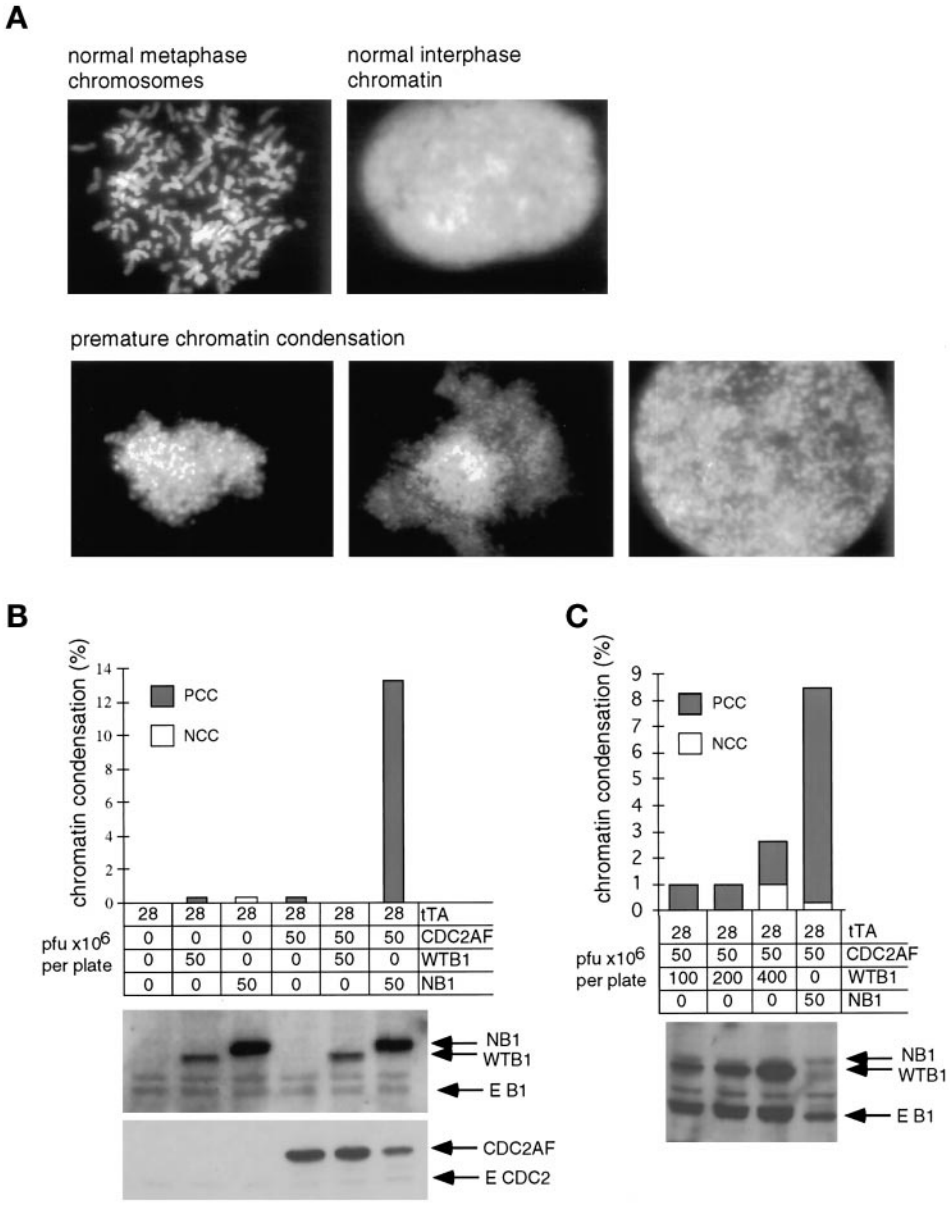

Figure 11. Chromatin condensation in TR9-7 cells arrested in G2 by p53, followed by expression of wild-type (WTB1) or nuclear-targeted (NB1) cyclin B1 and CDC2 T14A Y15F (CDC2AF). TR9-7 cells were released from a mimosine block in the absence of tetracycline, infected with recombinant adenoviruses, and analyzed using a chromosome-spreading technique. DNA was stained with Hoechst 33342. Cyclin and CDC2 expression is controlled by the tetracycline operator, and all samples were therefore infected with an adenovirus expressing the tetracycline activator (tTA), which drives expression from the tetracycline operator. (A) Examples of interphase and mitotic chromatin morphology and PCC. Cells were infected with adenoviruses 72 h after release from a mimosine block in the absence of tetracycline, and the morphology of chromatin was determined. (B) Percentage of cells exhibiting PCC or normal chromatin condensation (NCC) and expression of exogenous proteins. Three hundred cells in randomly selected fields were assessed for PCC and NCC as shown in A. Cells were infected with the indicated numbers of infectious virus particles, determined by infecting control HEK293 cells. Exogenous cyclin B1 was detected with a monoclonal antibody, and CDC2 T14A Y15F was detected with a monoclonal antibody to CDC2. Tagging of exogenous CDC2AF and wild-type cyclin B1 with the hemagglutinin and 9E10 epitope tags, respectively, reduces migration relative to that of the corresponding endogenous proteins. Addition of the T antigen nuclear localization signal to exogenous nuclear cyclin B1 (also tagged with 9E10) reduces its migration even further. The results shown represent at least two independent experiments. (C) PCC, NCC, and cyclin B1/CDC2 T14A Y15F expression in cells infected with different amounts of virus. Cells were infected and analyzed as described in A and B. EB1, endogenous cyclin B1; ECDC2, endogenous CDC2.

damage–induced G2 arrest, although less efficiently than did HPV-E6 (Thompson *et al.*, 1997). Attenuation of DNA damage–induced G2 arrest was also observed when p53 was eliminated via homologous recombination (Bunz *et al.*, 1998). In addition, p53-null human and mouse fibroblasts enter mitosis when DNA synthesis is blocked, whereas isogenic cells with p53 do not (Taylor *et al.*, 1999). Blocking the G2–M transition protects against the segregation of damaged or incompletely replicated DNA (Thompson *et al.*, 1997; Bunz *et al.*, 1998; Taylor *et al.*, 1999). We have now found that CDC2 activity is an important target of p53. It is also clear that p53-null cells are still inhibited from entering mitosis in response to damaged or unreplicated DNA (Kastan *et al.*, 1991; Taylor *et al.*, 1999), suggesting that p53-independent pathways also regulate the G2–M transition in response to stress.

Cells Arrested in G2 by p53 Have Low Levels of CDC2 Activity

Loss of CDC2 activity was associated with decreased levels of cyclin B1, detected 22 h after p53 induction, when many control cells were about to enter mitosis. However, the reduction in CDC2 activity was consistently larger than the reduction in cyclin B1 levels (Figures 2 and 4), suggesting

that p53 may cause a reduction in CDC2 activity in other ways. These observations are similar to those of Winters *et al.* (1998) in rat cells with functional p53 after exposure to ionizing radiation; the activity of CDC2 was decreased without changes in the levels of either CDC2 or cyclin B1. One potential explanation for these results is that p53 induces an inhibitor of CDC2. There is evidence that p21/waf1, a transcriptional target of p53, can regulate the G2–M transition. For example, overexpression of p21/waf1 can arrest tumor cells in G2 (Niculescu *et al.*, 1998). Because p21/waf1 is not an efficient direct inhibitor of the CDC2/cyclin B1 complex (Harper *et al.*, 1995), its role in mitosis may be indirect, possibly caused by inhibition of CDK2, required for maximal CDC2 activity in cycling *Xenopus* extracts (Guadagno and Newport, 1996). However, we did detect p21/waf1 in cyclin B1 immunoprecipitates from cells arrested in G2 by p53 (Figure 10). Further work is needed to determine the mechanism used by p21/waf1 to regulate the G2–M transition in human fibroblasts.

p16, another inhibitor of CDK/cyclin complexes, is induced at the G2–M boundary in response to UV radiation and may regulate entry into mitosis under these conditions (Wang *et al.*, 1996). Vogt *et al.* (1998) have shown that p16 is not expressed in the MDAH041 parents of TR9-7 cells because of promoter methylation. Therefore, p16 may not be required for induction of G2 arrest by p53. Interestingly, reversal of promoter methylation using 5-aza 2′-deoxycytidine leads to p16 expression and senescence of MDAH041 cells (Vogt *et al.*, 1998), suggesting that this cell line is a good model for normal fibroblasts.

We found that CDC2 activity was reduced when cells were arrested with a 4N DNA content in the presence of nocodazole and a high level of p53. The identification of a gene induced by p53 that encodes a microtubule-localized protein suggests that p53 might alter mitotic spindle function, leading to arrest with a 4N DNA content, possibly after an aborted attempt at mitosis (Utrera *et al.*, 1998). However, our results show that CDC2 is inactive in TR9-7 cells expressing p53 even in the presence of nocodazole and suggest that the cells have arrested in G2 before entering mitosis.

Direct observation by time-lapse microscopy of TR9-7 cells expressing p53 after release from a mimosine block indicated that they did not enter metaphase, which is normally seen as a change in morphology in which cells become round and refractile. Furthermore, our previous immunofluorescence analysis with DAPI-stained DNA showed that populations of TR9-7 cells arrested by p53 with a 4N DNA content have nuclei with a decondensed interphase morphology (Taylor *et al.*, 1999) (our unpublished data). This result suggests that the cells are in G2, consistent with time-lapse analysis and with our results with nocodazole-treated cells. We have also observed that the phosphorylation of histone H1b is greatly reduced in TR9-7 cells arrested with a 4N DNA content by p53 (Taylor *et al.*, 1999). Because high levels of phosphorylation of histone H1b are observed only in mitotic cells, the result is consistent with arrest in G2 (Taylor *et al.*, 1999). Also, chromosome spreading indicated that G2-arrested cells infected with control viruses had an interphase nuclear morphology, confirming that p53 blocks cells before they form mitotic chromosomes (Figure 11B). Our results with and without nocodazole show that p53 causes loss of CDC2 activity, an important part of the mechanism by which p53 causes G2 arrest.

p53 Represses the Transcription of cdc2 and *cyclin B1*

Overexpression of p53 caused loss of cyclin B1 followed by loss of CDC2. *cyclin B1* and *cdc2* mRNA levels were also reduced by p53 because of transcriptional repression. *cyclin B1* and *cdc2* mRNA abundance is reduced in cells treated with ionizing radiation in a p53-dependent manner (Azzam *et al.*, 1997; de Toledo *et al.*, 1998). Our results suggest that this effect is also caused by transcriptional repression by p53. *cyclin B1* promoter activity shows a gradual decrease in NIH3T3 cells arrested in S phase with aphidicolin (Nuckolls *et al.*, 1998). Blocking DNA synthesis with aphidicolin, hydroxyurea, or *N*-(phosphonacetyl)-L-aspartate induces p53 accumulation (Taylor *et al.*, 1999), and we now show that p53 represses the *cyclin B1* promoter. Therefore, repression of the *cyclin B1* promoter in NIH3T3 cells by aphidicolin may be caused by the induction of p53. We observed similar decreases of *cdc2* promoter activity and mRNA levels (~70% at 48 h), suggesting that promoter repression is the main mechanism leading to the downregulation of *cdc2* mRNA. However, *cyclin B1* mRNA decreased (~80% at 48 h) more than the promoter was repressed (~50% at 48 h), suggesting that posttranslational mechanisms may also contribute to the downregulation of *cyclin B1* mRNA by p53.

We have identified a region between −104 and −74 of the *cdc2* promoter required for repression by p53. This region contains a CAAT box, which activates transcription of this promoter (Sugarman *et al.*, 1995). Interestingly, a region further downstream, the R box, can interfere with transcription driven by the CAAT box in cells treated with phorbol ester (Sugarman *et al.*, 1995). Presumably, a protein that binds to the R box can block activation by one that binds to the CAAT box. It is possible that the repression by p53 is mediated by the R box. However, because there are no obvious p53 consensus–binding elements in the *cdc2* promoter, regulation is probably not attributable to direct binding of p53 to the promoter. Interestingly, the repression of topoisomerase II transcription by p53 has been localized to CAAT elements in the topoisomerase II promoter (Wang *et al.*, 1997). Repression of topoisomerase II may also contribute to G2 arrest by p53 because this enzyme is required to decatenate chromatin before chromosome condensation. However, it is not known whether overexpressed topoisomerase II can abrogate the G2 block induced by p53.

We also determined that repression of the *cyclin B1* promoter by p53 requires sequences between −123 and −287. This region contains two MyoD1 sites, an SP1 site, a USF site, and an E2F site, but no CAAT elements. As with the *cdc2* promoter, there is no obvious p53-binding element in the *cyclin B1* promoter. Therefore, repression is probably attributable to the effects of p53 on other proteins that are required for transcription of the *cyclin B1* promoter. For example, p53 may limit E2F activity via its induction of p21, which inhibits the CDK-dependent phosphorylation of Rb, which in turn inhibits E2F activity (Pietenpol *et al.*, 1994). This possibility is consistent with the presence of an E2F site in the region of the *cyclin B1* promoter required for repression.

The Decrease in CDC2 Activity Caused by p53 Is Not Attributable to Increased Phosphorylation of Threonine 14 or Tyrosine 15

Inhibitory tyrosine phosphorylation was decreased in cells arrested in G2 by p53 under conditions in which CDC2 activity was suppressed, consistent with observations made using a rat cell line containing activated *ras* and a temperature-sensitive allele of p53 (Leach *et al.*, 1998). p53 also caused a reduction in WEE1, the tyrosine kinase that phosphorylates CDC2 (Leach *et al.*, 1998). However, we did not observe any effect of p53 on WEE1 expression; perhaps this protein is regulated differently in the two cell types. The phosphorylation of CDC2 on tyrosine might be important for p53-dependent G2 arrest in some circumstances. p53 can transactivate the gene encoding *14-3-3σ* (Hermeking *et al.*, 1997), whose product is predicted to bind to and inhibit CDC25 if CDC25 is phosphorylated at serine 216 (Peng *et al.*, 1997). Several observations suggest that this mechanism of cell cycle inhibition is unlikely to occur in human fibroblasts. We observed that tyrosine phosphorylation of CDC2 was decreased upon G2 arrest and were not able to detect the 14-3-3σ protein in TR9-7 cells, even when the level of p53 was high. Previous studies have also failed to detect 14-3-3σ expression in fibroblasts (Hermeking *et al.*, 1997), even though it is expressed abundantly in a number of epithelial cell types (Leffers *et al.*, 1993). Furthermore, with a pan-reactive antibody against 14-3-3, none of the proteins recognized were induced by p53 in TR9-7 cells, and we obtained very similar results upon treatment of HT1080 cells with DNA-damaging agents. Therefore, the arrest of human fibroblasts in G2 in response to p53 does not appear to involve the upregulation of 14-3-3 proteins. Phosphorylation of CDC2 on tyrosine 15 occurs more efficiently when it is bound to cyclin B1 (Watanabe *et al.*, 1995). Therefore, the observed reduction in cyclin B1 levels may explain the reduction in tyrosine phosphorylation of CDC2.

Inhibition of CAK by p53 Is Not Necessary for G2 Arrest

Overexpression of p53 did not cause inhibition of CAK activity. Because of the importance of CAK in phosphorylating CDC2 on threonine 161 (Fisher and Morgan, 1994; Larochelle *et al.*, 1998), this result suggests that p53 may not modulate phosphorylation of threonine 161 in TR9-7 cells. Exposure to ionizing radiation leads to a reduction in CAK activity in normal but not p53-null mouse embryo fibroblasts (Schneider *et al.*, 1998). There are several potential reasons why we did not observe an effect of p53 on CAK. DNA damage induces the phosphorylation of p53 (reviewed in Giaccia and Kastan, 1998), but our studies involve overexpression of p53 without DNA damage. Perhaps modification of p53 is important for it to inhibit CAK. However, Schneider *et al.* (1998) did observe that a 16-fold molar excess of recombinant and presumably unmodified p53 inhibited recombinant CAK. Possibly not enough p53 is expressed in TR9-7 cells to inhibit CAK. Our results indicate that inhibition of CAK by p53 is not absolutely necessary for G2 arrest in human fibroblasts.

Targeting Cyclin B1 to the Nucleus Helps to Overcome G2 Arrest by p53

Cyclin B1 is essential for entry into mitosis (Murray *et al.*, 1989), and changes in its level have impact on this cell cycle transition. Overexpression of cyclin B1 shortens the DNA damage–induced delay in G2 (Kao *et al.*, 1997). Thus, loss of cyclin B1 in response to p53 overexpression might be sufficient to block entry into mitosis. However, overexpression of cyclin B1 alone did not efficiently overcome p53-dependent G2 arrest in TR9-7 fibroblasts. G2 arrest was abrogated when cyclin B1 was targeted to the nucleus, and this effect depended on the simultaneous expression of a form of CDC2 that cannot be inactivated by phosphorylation. At the highest level of exogenous wild-type cyclin B1, 2.6% of cells entered mitosis compared with 8.5% when nuclear-targeted cyclin B1 was expressed. However, under these conditions, wild-type cyclin B1 was greatly overexpressed compared with nuclear-targeted cyclin B1. This result suggests that, if expressed at a high enough level, wild-type cyclin B1 can induce mitosis in TR9-7 cells arrested in G2 by p53, but nuclear-targeted cyclin B1 is much more efficient. Innocente *et al.* (1999) showed that the *cyclin B1* promoter is repressed by p53 and that the G2 arrest induced by a temperature-sensitive p53 in a human ovarian tumor cell line can be abrogated by overexpression of wild-type cyclin B1 alone. Because Innocente *et al.* (1999) studied G2 arrest in a tumor cell line, it is possible that the requirement for nuclear targeting of cyclin B1 to overcome p53-dependent G2 arrest was lost during tumor development. Our observation that the abrogation of the p53-dependent G2 arrest by nuclear-targeted cyclin B1 requires coexpression of CDC2 T14A Y15F indicates that p53 might stimulate an additional pathway to reduce CDC2 activity, independent of the transcriptional repression of *cdc2* and *cyclin B1*. For example, we detected p21/waf1 in association with the cyclin B1/CDC2 complex in cells arrested in G2 by overexpression of p53. Perhaps p21/waf1 contributes to the inhibition of this complex. Thus, nuclear-targeted cyclin B1 may be unable to overcome G2 arrest unless CDC2 is expressed above the level bound by p21/waf1. It is not known why nuclear-targeted cyclin B1 is more efficient in overcoming G2 arrest compared with wild-type cyclin B1. Perhaps G2-arrested cells contain a cytoplasmic inhibitor of the CDC2/cyclin B1 complex, and targeting the complex to the nucleus overcomes such an effect. Other explanations are possible. Nonetheless, these experiments show that the CDC2/cyclin B1 complex is an important target for p53-mediated G2 arrest.

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