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Cyclin E Restores p53 Activity in Contact-Inhibited Cells

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The wild-type p53 protein is a potent growth suppressor when overexpressed in vitro. It functions as a transcriptional activator and causes growth arrest at the G_1/S stage of the cell cycle. We monitored p53 transactivation as an indicator of p53 function throughout the cell cycle. We first demonstrate that cells which exhibited contact inhibition of growth lacked p53 transactivation function at high cell density. Since these cells were noncycling, we examined whether the ectopic expression of any cyclin could override contact inhibition of growth and restore p53 transactivation function. The transfection of cyclin E at high cell density stimulated the progression of cells through the cell cycle and restored p53 transactivation function. The transcriptional activity of p53 induced by cyclin E was regulated at the level of DNA binding. Cells that did not show contact inhibition of growth had a functional p53 regardless of cell density. Thus, contact inhibition of cell growth corresponded to a lack of p53 transactivation function and the overexpression of cyclin E in these contact-inhibited cells stimulated cell cycle progression and resulted in p53 transcriptional activity.

The wild-type p53 protein is a growth and tumor suppressor. It suppresses transformation of cells in culture (13, 18), inhibits growth of tumor cells (1, 6, 9, 26, 40), and undergoes inactivating mutations in numerous human tumors (for a review, see reference 22), as well as in patients with Li-Fraumeni syndrome, an inherited predisposition to tumor development (37, 55).

Evidence to suggest that wild-type p53 is a transcriptional activator has accumulated. Wild-type p53-GAL4 fusion proteins (16, 44, 47), as well as p53 itself (15, 20, 30), activate transcription. Transforming mutants of p53 are inactive in transcriptional assays (7, 31, 46, 47). A consensus p53 DNA-binding sequence has been identified (11, 20). In addition, p53-responsive elements have been determined to be present in promoters of several genes, including the muscle creatine kinase, *GADD45*, *mdm2*, p53, and *WAF1*/*cip1* genes (3, 8, 12, 29, 56, 58, 61).

Wild-type p53 has been implicated in a variety of cellular processes, including cell cycle regulation. Many experiments suggest a role for p53 in growth arrest in late G_1. In quiescent mouse 3T3 cells, normal human T lymphocytes, and human diploid fibroblast cells, very low levels of p53 mRNA and protein are observed. Upon mitogenic stimulation, p53 mRNA and protein synthesis increases markedly, reaching peak levels in late G_1 (4, 48). In addition, overexpression of wild-type p53 arrests cells in late G_1, prior to the restriction point (9, 35, 38, 39). An increase in p53 activity and growth arrest in late G_1 is also seen upon UV or γ irradiation (28, 33, 45, 63). It has been suggested that p53 functions as part of a checkpoint that inhibits the G_1-to-S transition and that this activity may be necessary to allow repair of DNA damage or to prevent DNA replication when cells have a limited supply of nucleotide precursors (29, 33, 34, 36, 53, 60).

These examples indicate a function for p53 in late G_1. Since some cells in tissue culture are naturally arrested in G_1 upon contact inhibition, we examined the relationship between cell cycle progression and p53 transactivation function by using three cell lines that differ in their growth patterns at high cell density. Using promoters responsive to p53 activity, we showed that cells exhibiting contact inhibition of growth lacked p53 transactivation function at high cell density and were blocked in the early G_1 phase of the cell cycle. This loss of p53 function could be overcome by the cotransfection of cyclin E, but not cyclin A or B1. Cyclin E stimulated the progression of cells through the cell cycle in addition to restoring p53 transactivation function. Cells that did not show contact inhibition of growth had functional p53 at cell densities and were not blocked in G_1. We propose that cyclin E positively regulates p53 transactivation function by inducing cells to enter a window in the late G_1 stage of the cell cycle in which p53 is functionally active.

MATERIALS AND METHODS

Plasmids. Plasmid p0.7CAT has been described previously (51). Wild-type and mutant p53 expression plasmids, LTRXA and LTRKH, respectively, have also been described previously (24). SV40XA contains the simian virus 40 (SV40) enhancer and promoter, a p53 cDNA-genomic hybrid with p53 introns 2 and 3, and the SV40 polyadenylation signal. The cyclin expression plasmids encode complete cDNA sequences for human cyclins under the control of the cytomegalovirus promoter (25). The mdm2-luc plasmid was generated by blunt-end ligation of the 1-kb *Xho*I fragment containing the mdm2 promoter (27) to an *Sma*I-digested luciferase reporter (57).

Cell culture, DNA transfection, and reporter assays. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (NIH 3T3 cells), 10% fetal bovine serum (SaOS-2 cells), or 5% calf serum plus 5% fetal bovine serum (HeLa cells).

For DNA transfections, subconfluent cultures were plated at the appropriate cell density in 100-mm-diameter culture plates for 24 h prior to transfection. The reporter LTRXA (10 μg) or SV40XA (5 μg) and effector plasmids (5 μg) were cotransfected by calcium phosphate precipitation for 16 h (5). A β-galactosidase (β-Gal) expression plasmid (5 μg) was used in all transfections to normalize for transfection efficiency. At 40 h after transfection, cells were harvested and extracts were made. These were assayed for chloramphenicol acetyltransferase (CAT) activity (21) by using equivalent β-Gal units. The percent conversion of [^{14}C]chloramphenicol to acetyl and diacetyl chloramphenicol was quantitated by direct measurement of thin-layer chromatography plates with a Betagen betascanner. Luciferase assays were performed as described previously (57).

Immunoprecipitation and DNA binding. For immunoprecipitation, SaOs-2 cells were plated in triplicate at the appropriate cell densities and cotransfected with 10 μg of PBR322 or SV40XA, 2 μg of pSV2β-gal, and 5 μg of cyclin E. At approximately 48 h after transfection, one set of plates was metabolically labeled

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with [^{35}S]methionine and [^{35}S]cysteine for 2 h before harvesting. The second set of plates was harvested without labeling, and the cell lysates were subjected to β-Gal analysis. Labeled cell lysates showing equivalent β-Gal activities were immunoprecipitated with the appropriate p53 monoclonal antibodies and resolved on a sodium dodecyl sulfate–10% polyacrylamide gel as described previously (19). Nuclear extracts were prepared from the third set of plates for DNA binding assays. Aliquots of nuclear extracts containing equivalent amounts of β-Gal activity were immunoprecipitated with p53-specific monoclonal antibody pAb242 in the presence of labeled p53 consensus oligonucleotide as described previously (31).

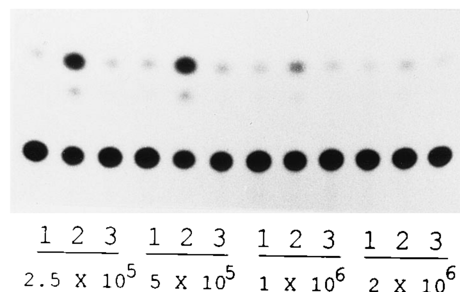
Flow cytometry. The proportions of cells in the different phases of the cell cycle were determined by flow cytometry. Cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS), and resuspended in PBS at 10^6 cells per ml. Cells were fixed by gradual addition of cold ethanol (75% final concentration) and stored at −20°C. After 24 h at −20°C, the fixed cells were deproteinized with 0.04% pancreatic pepsin in 0.01 N HCl for 30 min at 37°C. Following two washes with 3 ml of PBS, cells were resuspended in 1 ml of propidium iodide at 10 μg/ml in PBS containing 0.5% Tween 20 and 20 μg of RNase per ml. After 2 h of incubation in the dark, cells were analyzed in a FACSCAN flow cytometer (Becton Dickinson, San Jose, Calif.). Cell cycle distribution was analyzed with Cellfit software (Becton Dickinson) after all doublets and debris were sorted out. The DNA index was directly obtained as the ratio of the peak channel number of SaOS-2 cells to that of rat lymphocyte cells added as an internal standard.

RESULTS

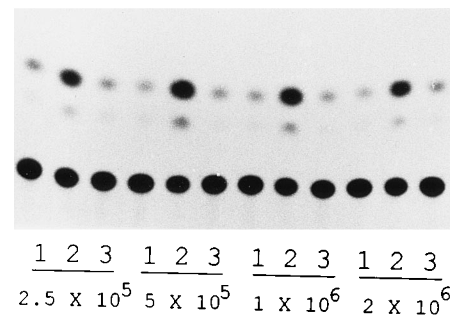
Wild-type p53 activation of the p53 promoter is cell density dependent in NIH 3T3 and SaOS-2 cells. Numerous experimental data suggest that p53 functions as a transcription factor to suppress transformation (31, 46, 47). Like many other transcription factors, wild-type p53 also regulates its own promoter. We have identified the *cis*-acting element in the p53 promoter that is activated by wild-type and not mutant p53 (8). This allows us to monitor autoregulation of the p53 promoter, and thus p53 function, rather than simply changes in mRNA or protein levels, which may not be indicative of a functional p53 protein. Since p53 levels vary during the cell cycle, with peak levels near the G_1/S boundary (38, 39), we reasoned that cell culture conditions that affect progression through the cell cycle may also modulate p53 function. Accordingly, we assayed for p53 function by its ability to activate the p53 promoter at different cell densities in three different cell lines, NIH 3T3, SaOS-2, and HeLa cells. Growth curves generated for these three cell lines showed that after attaining confluence NIH 3T3 and SaOS-2 cells exhibited markedly reduced growth rates, whereas HeLa cells continued to grow at the same rate (data not shown). The growth pattern of NIH 3T3 and SaOS-2 cells is characteristic of cells that exhibit contact inhibition of growth.

To determine the effect of cell density on p53 function, we monitored transcriptional autoregulation of the p53 promoter in transient transfection assays using wild-type or mutant p53 expression plasmids and a p53 promoter-CAT plasmid containing approximately 700 bp of p53 promoter sequences previously shown to be regulated by wild-type p53 (8). The mutant p53 used in these assays contains an in-frame linker insertion at amino acid 215 and is transcriptionally inactive (47). Transfections also included a β-Gal expression plasmid to measure and normalize for transfection efficiency. Cells were harvested at 40 h after transfection, β-Gal activity was measured, and CAT activity was determined. In SaOS-2 and NIH 3T3 cells, the p53 promoter was activated by wild-type p53 when cells were plated at low density but not when they were plated at a density of 10^6 cells per plate or higher (Fig. 1A and C). On the other hand, in HeLa cells, activation of the p53 promoter was independent of cell density (Fig. 1B and C). Wild-type p53 activity was sixfold higher than that of mutant p53 (Fig. 1C). The use of the SV40 promoter to express wild-type and mutant p53 in NIH 3T3 cells yielded identical results (data not shown).

A SaOs-2 cells



B HeLa cells



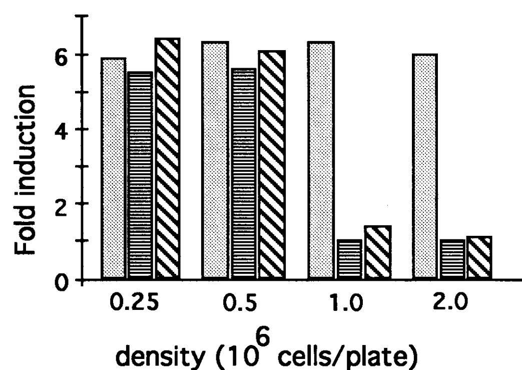


FIG. 1. Effects of cell density on p53 transcriptional autoregulation. Exponentially growing SaOS-2 cells (A) or HeLa cells (B) were plated into 100-mm-diameter tissue culture plates at the indicated cell numbers and transfected with the reporter plasmid p0.7CAT, containing the p53 promoter, and pBR322 (lanes 1), wild-type p53 (lanes 2), or mutant p53 (lanes 3). CAT activity was assayed as described in Materials and Methods after we normalized for transfection efficiency with a β-Gal expression plasmid. (C) The fold induction of the p53 promoter by wild-type p53 compared with mutant p53 in different cell lines as a function of cell density was quantified. Stippled bars, HeLa cells; horizontal stripes, NIH 3T3 cells; diagonal stripes, SaOS-2 cells.

These results suggested the presence of a functional p53 only at low cell densities in cells subject to contact inhibition.

To ensure that these results were not due to differences in transfection efficiencies at different cell densities, we examined the relationship between cell density and transfection efficiency in the three cell lines. A β-Gal plasmid (5 μg) under the control of the Rous sarcoma virus long terminal repeat promoter was transfected into HeLa, SaOS-2, and NIH 3T3 cells at different cell densities by calcium phosphate precipitation. As shown in Fig. 2A, the average β-Gal activity per cell for each cell line was not affected by cell density in several independent experiments. Transfections with pSV_2CAT, which

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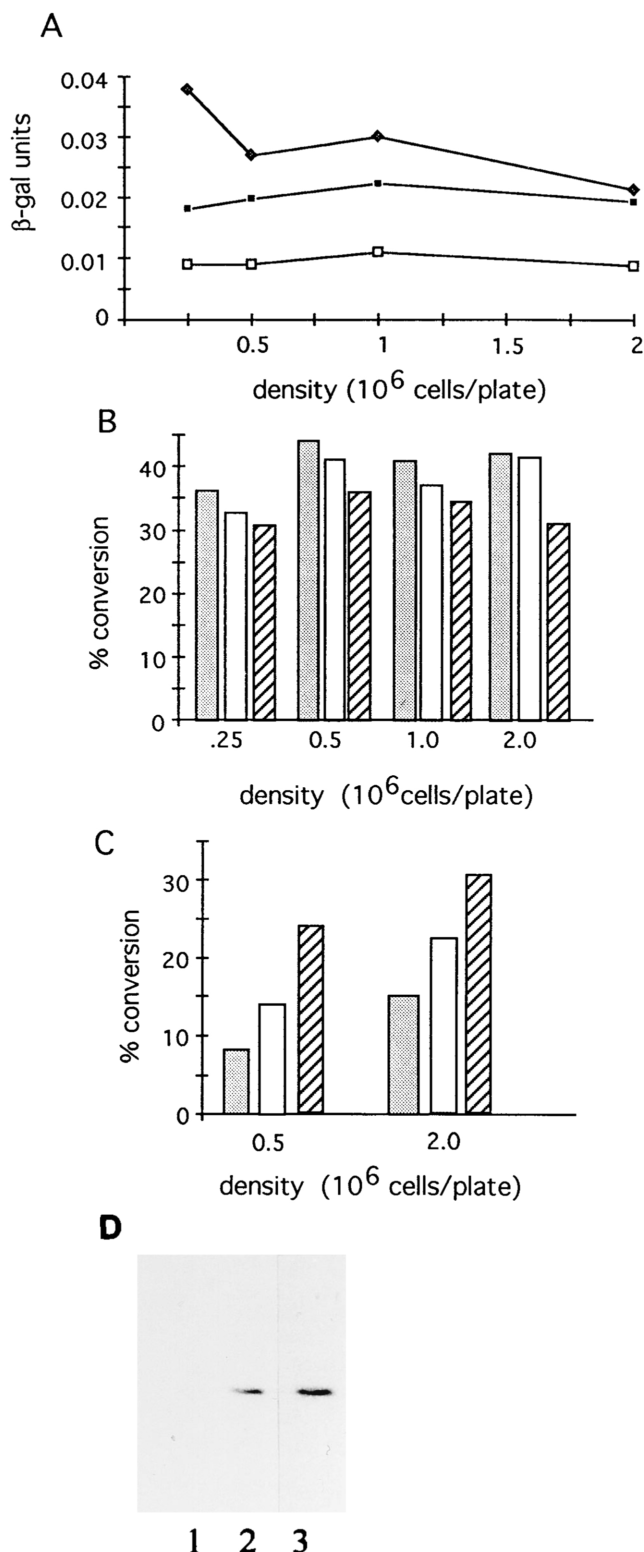

FIG. 2. Comparison of transfection efficiencies of cells plated at different cell densities. (A) Exponentially growing cells were plated onto 100-mm-diameter plates at 2.5×10^5, 5×10^5, 1×10^6, and 2×10^6 cells per plate and transfected with a β-Gal expression plasmid (5 μg). Transfected cells were harvested, and β-Gal activity was determined. The ordinate represents β-Gal units per 10^5 cells. Each point represents an average of at least three experiments performed in duplicate. Diamonds, NIH 3T3 cells; closed squares, SaOS-2 cells; open squares, HeLa cells. The plasmid pSV$_2$CAT (B), which contains the SV40 enhancer and promoter, or LTR-CAT (C) was transfected into HeLa cells (stippled bars), NIH 3T3 cells (open bars), and SaOS-2 cells (striped bars) at different cell densities. CAT activity was measured as the percent conversion. (D) A wild-type p53 expression plasmid (SV40XA) was transfected into SaOS-2 cells at low (lane 2) and high (lane 3) cell densities. After the cells were labeled with [^{35}S]Met and [^{35}S]Cys, they were harvested and immunoprecipitated with p53-specific monoclonal antibody pAb421. Lane 1 contains a pBR322-transfected control.

contains the SV40 promoter (21), yielded equivalent CAT activities for cells plated at low and high cell densities (Fig. 2B). In addition, to ensure that the murine sarcoma virus long terminal repeat, which regulates the expression of p53 in our p53 expression plasmids (24), was not affected by cell density, we transfected this murine sarcoma virus long terminal repeat promoter 5′ of the CAT gene (49) and assayed CAT activity at different cell densities. All assays were normalized for transfection using a β-Gal expression plasmid. The levels of CAT activity were similar at different cell densities, confirming that the murine sarcoma virus long terminal repeat was not affected by cell density (Fig. 2C). Thus, three different promoters analyzed as a function of cell density showed no significant variation in levels of expression of their reporters. Moreover, we measured ectopic p53 protein levels in SaOs-2 cells, since they lack endogenous p53 because of deletions. Cells at 5×10^5 (low cell density) or 2×10^6 (high cell density) per plate were transfected with pBR322 or a wild-type p53 expression plasmid (SV40XA) and labeled for immunoprecipitation experiments as described in Materials and Methods. The levels of p53 immunoprecipitated at low and high cell densities were comparable (Fig. 2D). These results suggest that the lack of wild-type p53 function in NIH 3T3 and SaOS-2 cells at high cell densities was due not to inefficient transfection or to the curtailment of transcription or translation of p53 but rather to a specific effect of high cell density on p53 function. Since the data for NIH 3T3 and SaOS-2 cells were comparable, in subsequent experiments we have often used NIH 3T3 cells for transactivation studies because they grow faster. We have limited the use of SaOS-2 cells to experiments that required the absence of endogenous p53.

Cyclin E overrides high cell density inhibition of p53 function in NIH 3T3 and SaOS-2 cells. Since p53 transactivation function was not seen in high-density cultures of NIH 3T3 and SaOS-2 cells, we examined the possibility that at high density these cells were noncycling, possibly blocked in the G_1 phase of the cell cycle at a point prior to p53 function. We first determined by flow cytometry the proportions of cells in the different phases of the cell cycle for cells plated at low and high cell densities. As depicted in Fig. 3, NIH 3T3 cells plated at high density (2×10^6 cells per plate) had a greater proportion of cells in G_1 than those cells plated at the low density of 5×10^5 cells per plate. Fluorescence-activated cell sorter (FACS) analysis of SaOS-2 cells produced the same profile (data not shown). At low density, approximately 50% of the cells were in G_1 (Fig. 3A), compared with 75% at high density (Fig. 3B). The corresponding decrease in the S and G_2/M phases is more apparent than the increase in G_1 at high cell density (Fig. 3B). On the other hand, HeLa cells showed equal distributions (50%) of cells in the G_1 phase of the cell cycle in low- and high-density cultures (Fig. 3C and D). Thus, high-density cultures of NIH 3T3 and SaOS-2 cells accumulate in G_1, a condition characteristic of high-density-induced quiescence.

The accumulation of cells in G_1 could affect the ability of wild-type p53 to function as a transcriptional activator in several ways. One possibility is that G_1 cells lack some cell cycle-regulated factors that are directly or indirectly required for p53 function. Experiments in a number of systems suggest that in

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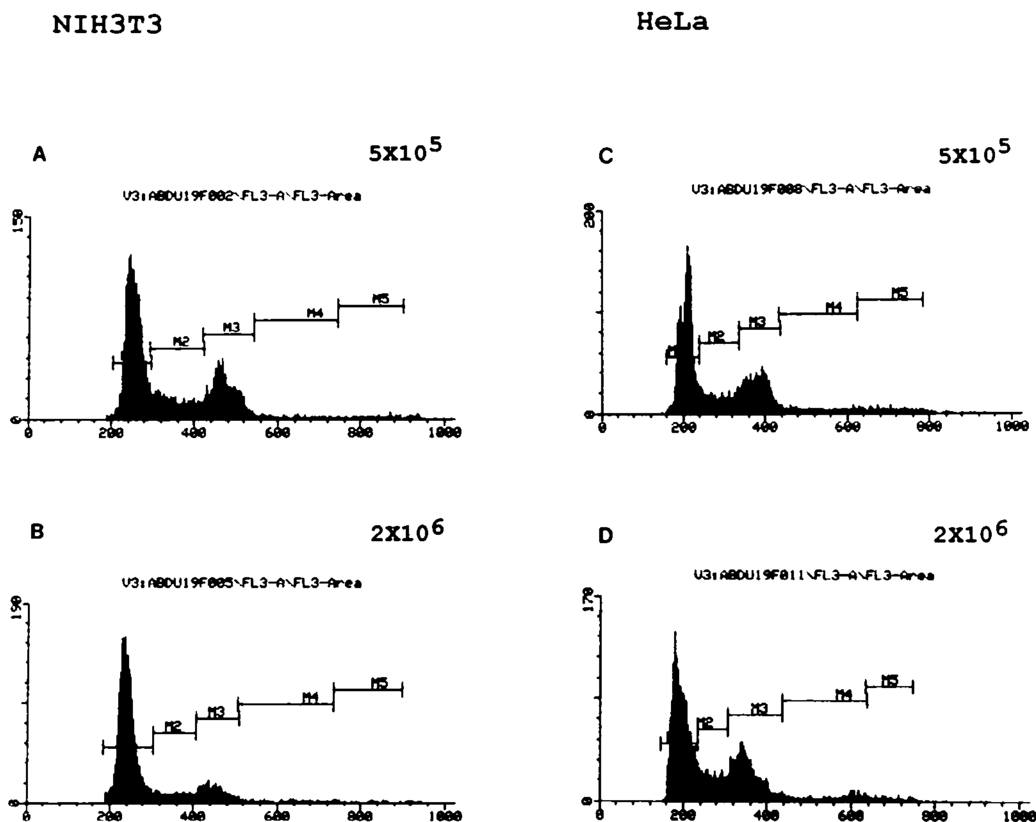

FIG. 3. Cell cycle analysis by flow cytometry of NIH 3T3 (A and B) and HeLa (C and D) cells plated at 5×10^5 (A and C) or 2×10^6 (B and D) cells per 100-mm-diameter plate. The number of cells is depicted on the vertical axis, and DNA content, as measured by propidium iodide intensity, is depicted on the horizontal axis. The M1 peak depicts the cells in G_1, M2 represents the number of cells in S, and M3 depicts cells in G_2/M. M4 and M5 are cell doublets.

higher eukaryotes cyclin-dependent kinases (cdks) are required for cell cycle progression (reviewed in references 42 and 54). Such kinases are themselves regulated by their binding to cyclins. Since cyclins are the cell cycle-regulated component of the cyclin-cdk complex, we investigated the effects of several cyclins on p53 transactivation function at high and low cell densities. NIH 3T3 or SaOS-2 cells at low (5×10^5 cells per plate) or high (2×10^6 cells per plate) densities were transfected with the reporter plasmid p0.7CAT, containing the p53 promoter, and with wild-type p53 or mutant p53 expression plasmids. In some samples human cyclin A (an S-phase cyclin), B1 (a mitotic cyclin), or E (a G_1/S cyclin) expression plasmids were also included. All cyclin expression plasmids used in these experiments have previously been shown to express their respective cyclins by immunoprecipitation with cyclin-specific antibodies (25). In both NIH 3T3 cells (Fig. 4A and B) and SaOS-2 cells (data not shown), the cotransfection of cyclin E with wild-type p53 activated the reporter at high cell density to a level that was comparable to that observed for activation at low cell density. In these experiments, cyclin E did not affect the basal activity of the p53 promoter (Fig. 4, lanes 1), nor did it have an effect in transfection experiments with mutant p53 (Fig. 4, lanes 3). The cotransfection of cyclin A or B1 with wild-type p53 also failed to alter the activation pattern of the p53 promoter. In addition, cotransfection with cyclin A, B1, or E did not alter the activation pattern of the promoter at low cell density (Fig. 4A). Transactivation by wild-type p53 in HeLa cells, which are not density arrested, was affected neither by cell density nor by cotransfection with any of the cyclins (Fig. 4C and D).

In order to further substantiate the finding that overexpression of cyclin E results in activation of p53 function, we assayed a different p53-responsive promoter under the same conditions. The mdm2 (murine double minute 2) promoter is activated by p53 in transfection experiments and in cell lines containing a temperature-sensitive p53 mutant upon induction of wild-type p53 activity (3, 27, 58). The p53 consensus element has been mapped just upstream of the second exon (2). NIH 3T3 cells at low (5×10^5 cells per plate) or high (2×10^6 cells per plate) densities were transfected with expression plasmids for mdm2-luciferase, wild-type or mutant p53, and β-Gal. After we normalized for transfection efficiencies with β-Gal, luciferase assays were performed (Fig. 5). Again, as in the previous experiment, the overexpression of cyclin E at high cell density rescued p53 transactivation function. At low cell densities the presence of cyclin E had no effect on p53 activity. Thus, the use of a different promoter which also contained a p53-responsive element led to the same results.

The next set of experiments were designed to study the levels of p53 protein and its DNA binding properties at low and high cell densities in the presence or absence of cyclin E. For these experiments, we used SaOS-2 cells, since they lack endogenous p53. SaOS-2 cells were transfected with a wild-type p53 expression plasmid (SV40XA) and with the cyclin E and β-Gal plasmids in triplicate. One set of plates was used to measure β-Gal activity in order to normalize for transfection efficiency. In another set of plates, cells were labeled and lysates were prepared. Immunoprecipitation of p53 was performed with monoclonal antibody pAb248. All plates whose contents were transfected with p53 showed the same amount of protein regardless

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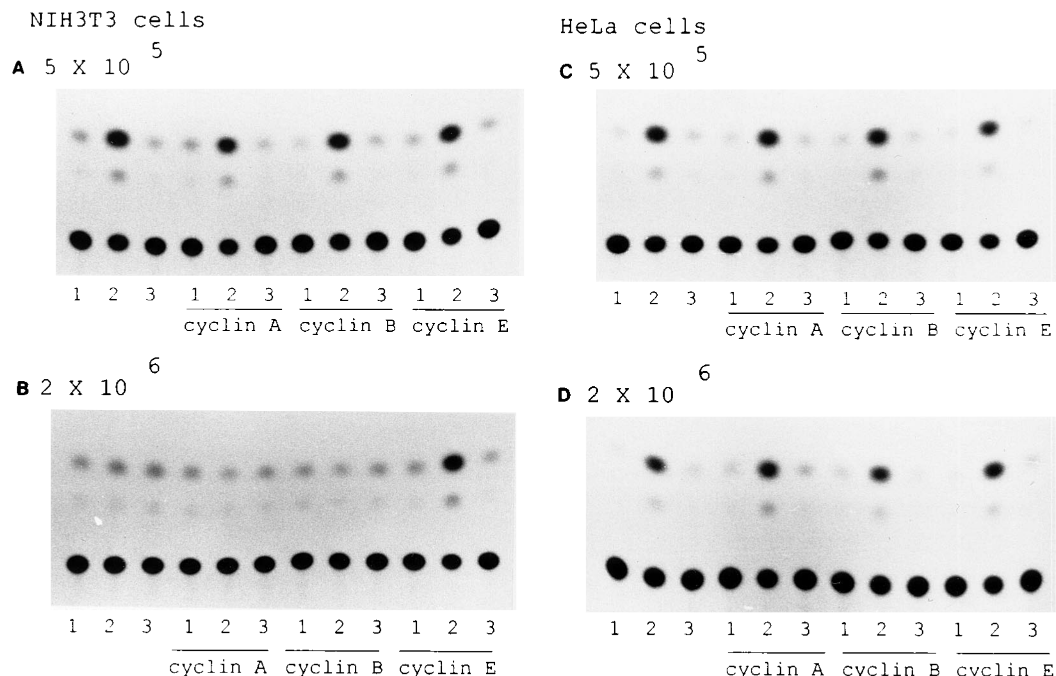

FIG. 4. Effect of cyclin cotransfection on wild-type p53 transcriptional regulation at low or high cell densities. Exponentially growing NIH 3T3 cells (A and B) or HeLa cells (C and D) were plated at 5×10^5 (A and C) or 2×10^6 (B and D) cells per 100-mm-diameter plate and transfected with reporter plasmid p0.7CAT, containing the p53 promoter, and control pBR322 (lane 1) or a wild-type (lane 2) or mutant (lane 3) p53 expression plasmid. The transfections which also included the human cyclin expression plasmids are labeled as such. Transfected cells were harvested, and CAT activity was measured.

of cell density or the presence of cyclin E (Fig. 6A). The third set of transfections was used to measure DNA binding. After transfection, p53 was immunoprecipitated with p53-specific monoclonal antibody pAb242 in the presence of labeled oligonucleotide containing a p53 binding sequence (Fig. 6B). At low cell density, transfections with a wild-type p53 expression plasmid resulted in specific DNA binding (Fig. 6B, lane 2). At high cell density, when p53 was transcriptionally inactive, it did not bind DNA (Fig. 6B, lane 3). However, at high cell density in the presence of cyclin E, p53 gained DNA binding function (Fig. 6B, lane 4). Taken together, these results indicate that cyclin E, a G_1/S-specific cyclin (10, 32), can override cell density-dependent inhibition of p53 DNA binding and transactivation functions.

Cyclin E induces G_1 progression in high-density cultures of NIH 3T3 and SaOS-2 cells. In mammalian cells, cyclin E functions as a regulator of the cdk2 kinase at the G_1-to-S transition (10, 32). In addition, cyclin E overexpression accelerates the transit of human fibroblasts and Rat-1 cells through G_1, pushing cells out of G_1 into S phase (43, 50). To determine whether cyclin E pushes density-arrested NIH 3T3 or SaOS-2 cells through G_1 to the point at which p53 is activated, we transfected these cells with the p53 promoter-CAT construct and cyclin E and p53 expression plasmids and analyzed by flow cytometry the distribution of cells in the cell cycle. When cells were plated at low density (5×10^5 cells per plate) and transfected with p53 expression plasmids or a pBR322 control in the absence of cyclin E, approximately 50% of the cells accumulated in G_1, with the remaining 50% being distributed about equally in S and G_2/M phases (Tables 1 and 2 for NIH 3T3 and SaOS-2 cells, respectively). In addition, at low cell density, cotransfection with the cyclin E expression plasmid did not affect the distribution of cells in the cell cycle (Tables 1 and 2). When NIH 3T3 or SaOS-2 cells at high cell density (2×10^6 cells per plate) were transfected with a p53 expression plasmid, about 75% of the cells accumulated in G_1 (Tables 1 and 2), a result remarkably similar to the results obtained without transfection (Fig. 3B). However, transfection of the cyclin E expression plasmid into the high-density cultures decreased the proportion of cells in G_1 from about 75 to 62% (Tables 1 and 2). A concomitant increase in the proportion of S-phase cells from approximately 10 to 17% was clearly visible for cells transfected with cyclin E compared with cells without cyclin E. Since on average 10% of the cells were transfected in these experiments (as measured by β-Gal staining), these values accurately reflect the transfection efficiencies. HeLa cells, which did not exhibit contact inhibition of growth, were not blocked in G_1 at high cell density and were not affected by cyclin E transfection

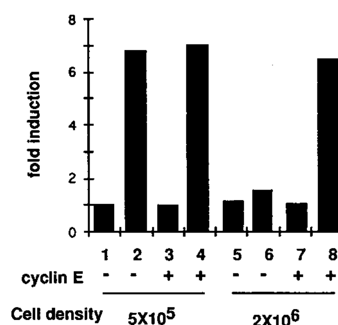

FIG. 5. Effects of cell density and cyclin E expression on a different p53-responsive promoter. The plasmid mdm2-luc, containing the mdm2 promoter, which is activated by p53, was transfected into NIH 3T3 cells at different cell densities (abscissa) with cyclin E and wild-type (even-numbered lanes) or mutant (odd-numbered lanes) p53 expression plasmids. Cells were harvested and assayed for luciferase activity after transfection efficiency was monitored.

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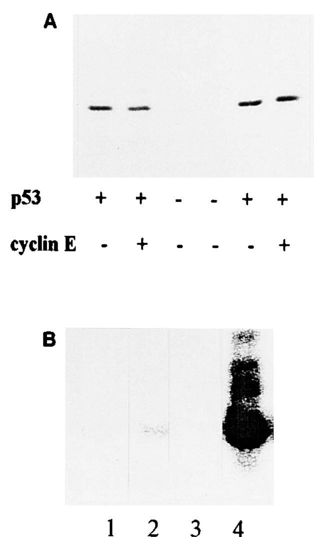

FIG. 6. p53 DNA binding activity and protein levels. (A) SaOS-2 cells were transfected with wild-type p53 (first two and last two lanes from the left) and cyclin E expression plasmids at 5×10^5 (first three lanes) or 2×10^6 (last three lanes) cells per plate. Following transfection, cells were labeled with [^{35}S]Met and [^{35}S]Cys. Cell lysates were immunoprecipitated with p53-specific monoclonal antibody pAb248. (B) Lysates from SaOS-2 cells transfected with the pBR322 control (lane 1) or with p53 expression plasmids at 5×10^5 (lane 2) or 2×10^6 (lanes 3 and 4) cells per plate with (lane 4) or without (lane 3) cyclin E were immunoprecipitated with p53 antibody pAb242 and ^{32}P-labeled p53 consensus sequence according to published procedures (31). Immunoprecipitated complexes were treated with protease, and the bound DNA was run on a polyacrylamide gel.

(Fig. 3 and data not shown). We have made numerous attempts to isolate only the transfected cells by using the cell surface marker CD20. However, the transfection of CD20 appears to result in cell death. At the time of harvest the cells are not confluent and therefore are not representative of previous high-cell-density experimental conditions. In summary, it appears that cyclin E overrides the inhibitory effect of high cell density on wild-type p53 transactivation function by inducing cells to resume cycling out of the G_1 block.

TABLE 1. Analysis of the distribution of NIH 3T3 cells in the cell cycle by flow cytometry after transfection with various effectors

Cell density and construct(s)[a]	% of cells in:		
	G_1	S	G_2/M
5×10^5 cells/plate			
pBR322	48	22	24
wt p53	50	21	27
mut p53	49	23	25
pBR322 + cyc E	47	20	26
wt p53 + cyc E	49	23	25
mut p53 + cyc E	46	21	25
2×10^6 cells/plate			
pBR322	74	10	10
wt p53	76	9	12
mut p53	74	10	12
pBR322 + cyc E	60	17	14
wt p53 + cyc E	62	17	13
mut p53 + cyc E	63	16	12

[a] In addition, the reporter p0.7CAT was transfected in all experiments so that CAT assays and FACS analyses could be performed in the same experiment. wt, wild type; mut, mutant; cyc, cyclin.

TABLE 2. Analysis of the distribution of SaOS-2 cells in the cell cycle by flow cytometry after transfection with various effectors

Cell density and construct(s)[a]	% of cells in:		
	G_1	S	G_2/M
5×10^5 cells/plate			
pBR322	53	21	22
wt p53	54	23	23
mut p53	49	21	29
pBR322 + cyc E	51	24	28
wt p53 + cyc E	50	26	23
mut p53 + cyc E	53	22	21
2×10^6 cells/plate			
pBR322	79	10	7
wt p53	75	9	14
mut p53	74	10	11
pBR322 + cyc E	65	18	14
wt p53 + cyc E	68	16	10
mut p53 + cyc E	66	18	11

[a] In addition, the reporter p0.7CAT was transfected in all experiments so that CAT assays and FACS analyses could be performed in the same experiment. wt, wild type; mut, mutant; cyc, cyclin.

DISCUSSION

Numerous studies have shown that the ability of p53 to suppress growth is correlated with its ability to modulate transcription via sequence-specific DNA binding (20, 30, 62). Two promoters activated by p53 in transient transfection assays are the p53 and mdm2 promoters (8, 27, 58). In the present study, we used such a functional assay to investigate the relationship between p53 function and cell cycle progression. Using cell lines that differ in their growth responses to high cell density, we showed that in NIH 3T3 and SaOS-2 cells, cell lines that exhibited contact inhibition of growth and accumulated in G_1 at high cell density, wild-type p53 did not function as a transcriptional activator at high cell densities. In contrast, in HeLa cells, which did not exhibit contact inhibition of growth at high cell density, wild-type p53 was functional at all cell densities tested. This is the first study indicating that p53 transactivation and DNA binding properties are specific to the G_1/S phase of the cell cycle.

The expression of cyclin E released the G_1 block, allowing high-density cultures of NIH 3T3 and SaOS-2 cells to progress through G_1 into S and G_2/M phases of the cell cycle. Furthermore, the cotransfection of cyclin E into NIH 3T3 and SaOS-2 cells at high cell density restored p53 transactivation function. A twofold increase in p53 transactivation function by cyclin E has previously been reported for SaOS-2 cells transfected at an intermediate density of 10^6 cells per plate (52). The fact that cyclin E overexpression at low cell densities did not affect cell cycling or increase p53 activity could simply mean that cofactors of cyclin E, perhaps cdk2, were already bound to endogenously expressed cyclin E and were therefore limited. At high cell density, when cells are not cycling, cdk2 would be available for binding to exogenously expressed cyclin E. The overexpression of wild-type p53 in these cells did not appear to suppress growth, most likely because the p53 was tied up in activating the CAT reporter construct and not endogenous targets.

At high cell densities, both NIH 3T3 and SaOS-2 cells showed contact inhibition of growth. It is likely that the lack of p53 transactivation function at high cell density was a reflection of the absence of cells at the point in late G_1 at which p53 acts. In support of this possibility, HeLa cells, which are not subject to contact inhibition of growth or to accumulation in G_1,

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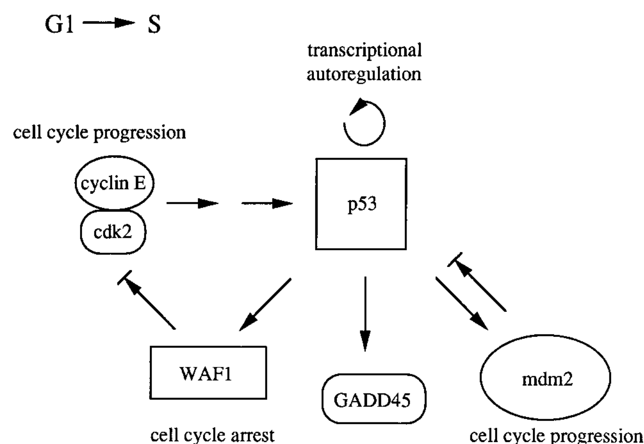

FIG. 7. p53 and cell cycle regulation. A model of the events at the G_1/S boundary centered around p53. The cyclin E-cdk2 complex activates p53, which in turn down-regulates cyclin E-cdk2 by transactivation of the inhibitor p21 in an autoregulatory loop. Activation of p53 allows transcription of *GADD45* and itself. Subsequently, p53 turns on *mdm2*, which feeds back and inhibits p53 function. Down-regulation of p53 then allows cell cycle progression.

showed p53 transactivation function at all cell densities tested. Additional support for the hypothesis that only cells in late G_1 express a functional p53 is provided by analysis of the effects of overexpression of p53 on cell cycle progression. Cells overexpressing a temperature-sensitive p53 mutant were synchronized and shifted to a permissive temperature for analysis of wild-type activity (38). Overexpression of p53 did not suppress growth at any stage of the cell cycle except at the G_1/S boundary. These cells probably remain arrested at G_1/S because of p53 overexpression. They cannot down-regulate p53 to proceed to the next stage. Thus, in a normal cell cycle, the activation of p53 would result in a transient signal to stop growth, i.e., it would serve a cell cycle checkpoint function.

The major observation that we have made is that cyclin E expression restores p53 transcriptional activation in contact-inhibited cells. This transcriptional activation of p53 by cyclin E is regulated at the level of DNA binding. It may seem paradoxical that cyclin E, a positive regulator of cell cycle progression, stimulates the activity of wild-type p53, a negative regulator of growth. This relationship, however, is not necessarily contradictory, especially if these proteins are active at different times, ensuring a balanced and proper progression through the cell cycle. Cyclin E activity pushes cells through one part of the cycle into the next. This next stage requires p53 function to suppress growth temporarily while the cell assesses its status and capacity to proceed. This window allows time for the cell to repair damaged DNA before it replicates its DNA in S phase, a function that has been attributed to p53 because of its ability to activate the *GADD45* gene (29, 63).

Since p53 functions as a transcriptional activator, what targets does p53 activate and at what time? Our model predicts that p53 activates a series of targets in an orderly manner (Fig. 7). First, cyclin E allows activation of p53 function perhaps by phosphorylation. We have been unable to detect a direct interaction of cyclin E-cdk2 with p53 by using a baculovirus system that overexpresses all three proteins. An early target of p53 transactivation in our model is WAF1/Cip1 (12), an inhibitor of the cyclin E-cdk2 complex (23, 59). Inactivation of cyclin E ensures a pause in cell cycle progression in G_1/S. The next target of p53 transactivation may be the *GADD45* gene, which is involved in DNA repair (29). This activation allows the cell to repair any DNA damage before proceeding. It is reasonable to speculate that positive regulators of G_1 progression trigger p53 function because they enhance the transition of cells into the substage of G_1 in which p53 functions as "guardian of the genome" (34). During this time p53 also has the ability to regulate its own expression. The final target of p53 transactivation in our model is the *mdm2* gene (3, 14, 58), whose product has transforming ability (17). mdm2 in turn inactivates p53 function, forming an autoregulatory loop (3, 41, 58). Released from inhibition, the cell replicates its DNA and continues toward G_2/M checkpoints.

How does p53 manage to activate all these genes at just the right time, in precisely the correct order? One possibility is that p53 forms complexes with other proteins to activate specific targets. This hypothesis is supported by the fact that the p53 consensus response element, 5′PuPuPuC(A/T)(T/A)GPy PyPy3′ (11), is not well conserved. In addition, comparison of this sequence with those of p53-responsive elements present in various promoters never shows a complete match. The lack of conservation of the p53 DNA-responsive element suggests that other factors may be required to achieve specificity. This hypothesis also predicts that other proteins complexing with p53 should be activated at precisely the right time.

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