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Inverse Regulation of Cyclin B1 by c-Myc and p53 and Induction of Tetraploidy by Cyclin B1 Overexpression[1]

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ABSTRACT

We have shown previously that mitotic spindle inhibitors allow the c-Myc oncoprotein to uncouple mitosis from DNA synthesis, resulting in the acquisition of tetraploidy. This can also occur in the absence of spindle inhibition if c-Myc deregulation is combined with inactivation of the p53 tumor suppressor. Under these conditions, cyclin B1 protein is induced but retains its normal cell cycle regulation. We now show that the cyclin B1 promoter is directly but oppositely regulated by c-Myc and p53. Enforced expression of cyclin B1 also induces tetraploidy, either after mitotic spindle inhibition or in the absence of such inhibition if cyclin B1 is coexpressed with c-Myc. Cyclin B1 represents a new class of c-Myc target genes that is also regulated by p53. It is also the first identified downstream effector of c-Myc able to produce the chromosomal instability that characterizes virtually all tumor cells.

INTRODUCTION

Deregulation of the *c-Myc* oncogene occurs frequently in human cancers (1). c-Myc protein overexpression can immortalize cells, reduce their growth factor requirements, promote cell cycle progression, and inhibit differentiation (2–5). c-Myc may also promote the genomic instability that typifies malignant tumors (6). Initial reports described the c-Myc-mediated amplification of specific chromosomal loci, including the genes for dihydrofolate reductase, cad, and cyclin D2 (7–11). We recently demonstrated that treatment with mitotic spindle inhibitors, or concurrent inactivation of the p53 tumor suppressor protein, causes a more profound form of c-Myc-mediated genomic instability, namely the generation of tetraploidy (12). In the latter case, this occurs in the absence of spindle inhibition and is associated with a marked increase in cyclin B1 and cdc2 kinase activity, without a concomitant increase in cdc2 protein levels. The normal cell cycle regulation of cyclin B1, however, is retained.

Elevated levels of cyclin B1 often precede the onset of tumor cell immortalization and aneuploidy (13–15). Cyclin B1 has also been reported to be negatively regulated by p53 (16–18). We have now explored the possibility that the *cyclin B1* gene is also a direct c-Myc transcriptional target and that cyclin B1 deregulation underlies the acquisition of tetraploidy. We show here that c-Myc and loss of p53 cooperate to induce cyclin B1 mRNA and protein. This novel regulation of cyclin B1 identifies it as the prototype for a new class of c-Myc target genes. The importance of cyclin B1 as a c-Myc transcriptional target is additionally underscored by the ability of cyclin B1 overexpression alone to sensitize cells to the acquisition of tetraploidy.

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MATERIALS AND METHODS

Plasmids and Cell Lines. The plasmids pSVLneo, pSVLneo-c-Myc, and pLTRtsp53-Hygro have been described previously (12, 19, 20). The latter vector encodes the temperature-sensitive p53 protein containing an Ala135 → Val mutation (19). pAPuro-cyclin B1 was constructed by ligating a 1.5-kb *Sma*I fragment containing the coding region of human cyclin B1 into the blunt-ended *Eco*RI site of the pAPuro vector (12). The cyclin B1 promoter E-box was mutated as described previously (21). Luciferase reporter vectors were constructed by PCR-mediated amplification of WT or mutant cyclin B1 promoter segments between −1075 bp and +52 bp relative to the transcriptional start site. PCR primers contained an engineered *Bam*HI site on the forward primer and a *Bgl*II site on the reverse primer. After digestion with *Bam*HI and *Bgl*II, amplified fragments were cloned into the *Bgl*II site of the pGL2-luciferase reporter vector (Promega, Inc., Madison, WI). DNA sequencing confirmed the orientation and identity of each plasmid. For stable transfection of 32D cells, 5–10 μg of each vector along with 1–2 μg of pCMV-β-gal-Puro was linearized in the plasmid backbone, followed by electroporation into 2×10^7 cells (12). Two days later, puromycin (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1 μg/ml After 10–14 days, the resultant puromycin-resistant clones were pooled. β-galactosidase and luciferase assays were then performed as described previously (20). Rat1a transfections were performed similarly except that individual clones were selected. The results shown here were obtained with a single clone but were confirmed with two additional independently derived clones.

Cell Cycle Studies. Cell cycle analyses were performed as described previously (12). Briefly, cells were washed twice in ice cold PBS and then resuspended in 1–2 ml of 10 mM NaCl, 10 mM Tris-HCl, (pH 8.0), 0.1% NP40, 10 μg/ml RNase A, and 15 μg/ml propidium iodide (all from Sigma Chemical Co.). Cell cycle analyses were performed on a Becton Dickinson FACStar fluorescence-activated cell sorter. Cells (2×10^4) were analyzed for each assay. Quantitation was performed using single histogram statistics (12).

RNA and Protein Analyses. Northern blotting was performed as described previously (20, 21). DNA probes consisted of the above cyclin B1 cDNA fragment, a 0.7-kb rat GAPDH[3] cDNA, and a 1.5-kb human c-Myc cDNA containing the entire coding region. Immunoblotting was performed as described previously (12). Cdc2-directed histone H1 kinase activity was determined on cyclin B1 immunoprecipitates as described previously (12, 22).

Nuclear Run-on Assays. These were performed as described (23). Briefly, nuclei were prepared from $\sim 5 \times 10^7$ cells and incubated at 30°C for 30 min in the presence of 50 μCi of [α-^{32}P]-UTP (specific activity 3000 Ci/mmol; New England Nuclear, Boston, MA). After purifying the labeled nascent RNA transcripts over G-50 Sephadex spin columns, they were precipitated and redissolved in a minimal volume of hybridization buffer [300 mM NaCl, 10 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (pH 7.4), 1 mM EDTA, 0.2% SDS, 1 × Denhardt's solution, and 250 μg/ml *Escherichia coli* RNA]. Labeled RNA ($\sim 5 \times 10^6$ dpm/ml) was hybridized to nitrocellulose strips onto which had been dot-blotted duplicate 2-μg aliquots of either pAPuro-cyclin B1, the empty pAPuro vector, or a plasmid containing a GAPDH cDNA. The latter two vectors served as negative and positive controls, respectively, for the specificity of the hybridization reaction. Hybridizations were performed at 68°C for 48 h. After exhaustive washing, the blots were exposed to BioMax MS film with a BioMax intensifying screen (Kodak, Rochester, NY).

[3] The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; WT, wild-type; CHX, cycloheximide; cdk, cyclin-dependent kinase; ds, double-stranded.

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EMSAs. Synthetic oligonucleotides consisted of the WT human cyclin B1 promoter sequence 5′-GAGGCAGAC*CACGTG*AGAGCCTGG-3′ or the mutant sequence 5′-GAGGCAGAC*CTCGAG*AGAGCCTGG-3′, where italicized bases denote the WT or mutant E-box. Each oligonucleotide (100 ng) was end labeled with T4 polynucleotide kinase (New England Biolabs, Beverley, MA) and 50 μCi of [γ-^{32}P]ATP (specific activity >3000 Ci/mmol; New England Nuclear) and then annealed with a 10-fold molar excess of the unlabeled complementary strand. Recombinant c-Myc and Max(S) proteins were purified to >90% purity using nickel-agarose affinity chromatography (24). Max(S) is the 151 amino acid isoform of Max that binds DNA only as a heterodimer in association with c-Myc (24). EMSAs were performed with ~20 pg of [^{32}P]-labeled ds oligonucleotide (specific activity ~5×10^8 dpm/μg). Recombinant proteins (~20 ng of each) were added to a final volume of 20 μl in binding buffer and incubated at 40°C for 20 min before electrophoresis.

RESULTS

Cyclin B1 Levels Are Inversely Regulated by c-Myc and p53. We used two cell lines in which the consequences of c-Myc overexpression have been studied previously (12). In 32D myeloid cells, c-Myc accelerates apoptosis after growth factor withdrawal (25, 26) and cooperates with loss of p53 to promote the acquisition of tetraploidy after 12–20 weeks of logarithmic growth (12). In Rat1a fibroblasts, c-Myc overexpression also accelerates apoptosis in the absence of serum, confers anchorage-independent growth, and can also promote the acquisition of tetraploidy in response to mitotic spindle inhibition (27–29).

32D cells, stably transfected with a c-Myc expression vector (32D-c-Myc cells) or a control plasmid (32D-neo cells; Ref. 12), or Rat1a cells, expressing a modified c-Myc-estrogen receptor fusion protein (Rat1a-MycER cells; Ref. 30), were transfected again with pLTRtsp53-hygro (19) or the control vector. All three cell lines expressed high levels of p53 protein (Fig. 1*A*).

The above cell lines were examined for cyclin B1 expression under conditions where p53 was maintained in either the WT or mutant state (32°C and 38°C, respectively). In 32D cells, the largest increase in cyclin B1 protein levels was seen in c-Myc/tsp53 cells maintained at 38°C (Fig. 1*B, Lane 3*). The dependence on mutant p53 for sustained expression of cyclin B1 was confirmed when these cells were temperature shifted to 32°C, at which point cyclin B1 rapidly disappeared (*Lanes 9–12*).

Similar results were obtained with Rat1a-mycER/tsp53 cells. Although the activation of c-Myc alone (Fig. 1*C, Lanes 1–4*), or the inactivation of p53 alone (*Lanes 5–7*) each resulted in some induction of cyclin B1, the greatest induction was observed with the combination of these two manipulations (*Lanes 8–10*).

Northern blots confirmed the above results. At 32°C, both 32D-neo/tsp53 cells and 32D-c-Myc/tsp53 cells expressed barely detectable levels of cyclin B1 transcripts (Fig. 1*D, Lanes 1* and *2*). While shifting to 38°C, cyclin B1 transcripts increased somewhat in 32D-neo/tsp53 cells, paralleling the inactivation of p53 (*Lanes 3* and *4*). CHX (*Lanes 5* and *6*) inhibited cyclin B1 transcript induction, perhaps reflecting the ongoing requirement for the short-lived endogenous c-Myc protein. In contrast, reculturing 32D-c-Myc/tsp53 cells at 38°C caused a marked up-regulation of cyclin B1 transcripts (*Lanes 7* and *8*), even in the presence of CHX (*Lanes 9* and *10*). On the basis of phosphorimager quantitation, the degree of cyclin B1 transcript up-regulation varied, depending on the experiment, between 10- and 30-fold in comparison with that seen in the presence of c-Myc overexpression or p53 inactivation alone.

Studies using Rat1a-MycER/tsp53 cells confirmed these findings and also demonstrated the direct contribution of c-Myc to cyclin B1 induction. As seen in Fig. 1*E,* either the activation of c-Myc or the inactivation of p53 resulted in only a modest induction of cyclin B1 transcripts (compare *Lanes 2–6* and *7–11* with *Lane 1*). The combination of c-Myc activation and p53 inactivation, however, markedly induced cyclin B1 transcripts (*Lanes 12–14*) even in the presence of CHX (*Lanes 15* and *16*). Together with the experiments presented in Fig. 1, *B–D,* these results are consistent with the interpretation that c-Myc and mutant p53 act directly and cooperatively to induce cyclin

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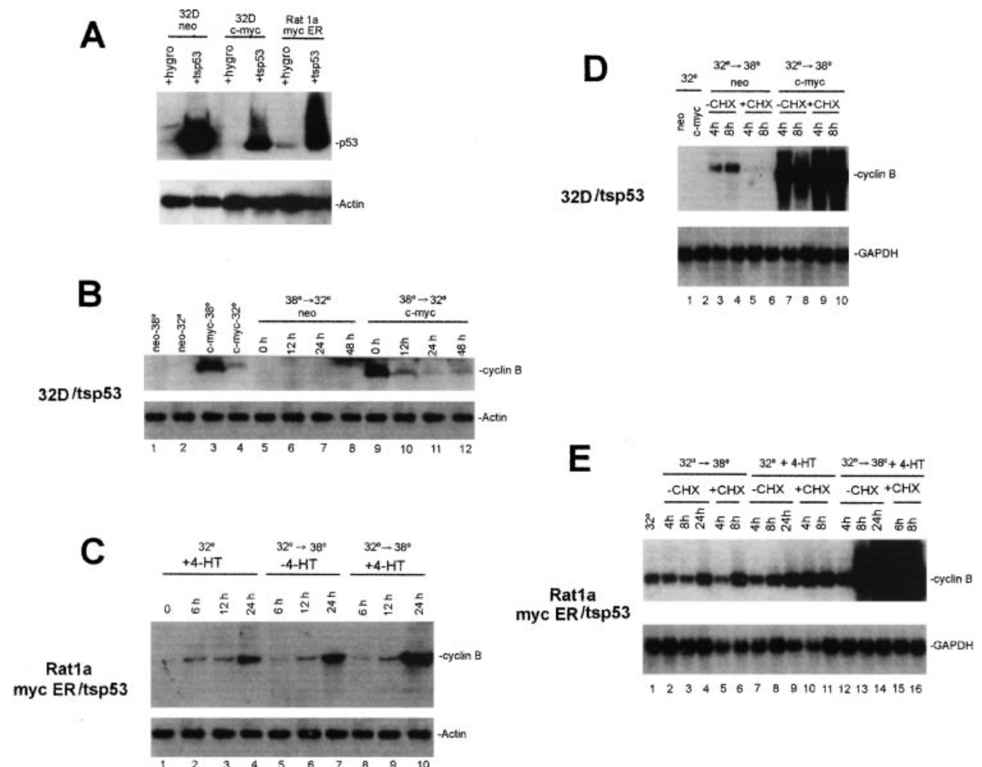

Fig. 1. Characterization of 32D and Rat1a cell lines. *A,* tsp53 levels. 32D-neo, 32D-c-Myc, or Rat1a-MycER cells were transfected with a tsp53 expression vector or with the empty hygromycin control vector. Stable transfectants were then assayed for the expression of p53 protein (*top panel*) or actin (*bottom panel*) by Western blotting. *B,* cyclin B1 levels in 32D cells. Each of the above 32D cell lines was maintained continuously at 38°C or at 32°C for 24 h (*Lanes 1–4*) or switched from 38°C to 32°C for the indicated periods of time (*Lanes 5–12*). Cell lysates were then analyzed for cyclin B1 protein or actin. *C,* cyclin B1 levels in Rat1a-MycER cells. Each of the above Rat1a-MycER cell lines was maintained at 32°C for 24 h (*Lane 1*) before shifting to 38°C for the indicated periods of time. In some cases, MycER was activated by the addition of 4-HT to a final concentration of 250 nM (*Lanes 2–4* and *8–10*). *D,* Northern analyses of 32D cells. 32D-neo/tsp53 or 32D-c-Myc/tsp53 cells were maintained at 32°C for 24 h (*Lanes 1* and *2*) or shifted from 32°C to 38°C for the indicated periods of time (*Lanes 3–10*). Where indicated, CHX (final concentration 10 μg/ml) was added to the cultures at the time of the temperature shift. Northern blots were probed sequentially with cyclin B1 (*top panel*) or GAPDH (*bottom panel*) cDNA probes. *E,* Northern analyses of Rat1a-MycER/tsp53 cells. The cell line was kept at 32°C for 24 h (*Lane 1*) or switched to 38°C for the indicated times. 4-HT was added either with or without the concurrent addition of CHX as described above. Total RNA blots were again hybridized with cyclin B1 or GAPDH probes.

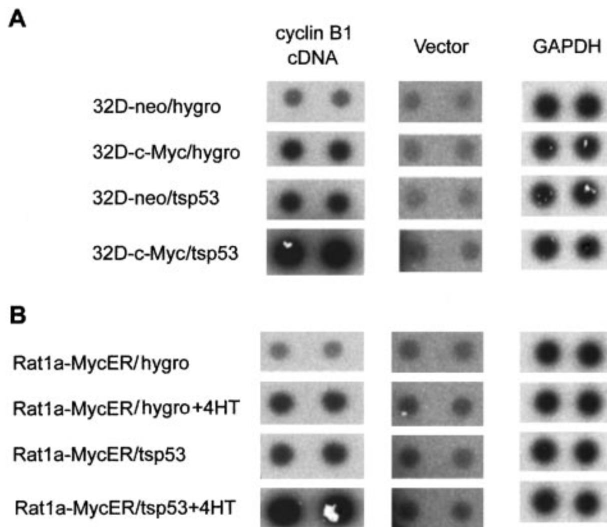

Fig. 2. Nuclear run-on assays in Rat1a and 32D cells. In *A,* the indicated 32D cell lines were maintained in log phase growth and then placed at 32°C for 12 h to allow p53 to assume its WT conformation. At the end of that time, the cells were recultured at 38°C for 4 h., harvested, and prepared for nuclear run-ons (23). In *B,* the indicated Rat1a cell lines were cultured as described for 32D cells except that 4-HT was added to a final concentration of 250 ng/ml at the time of the 32°C–>38°C temperature shift. Cells were then harvested for nuclear run-ons. In both cases, ^{32}P-labeled transcripts were hybridized with nitrocellulose filters spotted with duplicate aliquots of pAPuro-cyclin B1, control pAPuro plasmid DNA, or a control GAPDH cDNA plasmid.

B1 in two different cell types. The discordance between cyclin B1 RNA and protein levels in some experiments may reflect the multiple levels of its transcriptional, translational, and posttranslational regulation (31–34).

It was possible that some of the differences in cyclin B1 protein and mRNA seen in the various cell lines were the result of changes in cell cycle profiles or rates of apoptosis, particularly in instances where the cells have been incubated at 32°C. Therefore, we compared the cell cycle profiles of each of the 32D and Rat1a cell lines shown in Fig. 1, either during log phase growth at 38°C or after 16 h at 32°C. In the former case, we confirmed the absence of any significant differences of 32D cell cycle profiles (12) and showed that this also applied to Rat1a cells. In the latter case, incubation at 32°C resulted in <2-fold reductions in the S and G_2-M populations in all cases. All cell lines also remained highly viable (>88%) during the 32°C incubation period (data not shown). Thus, we conclude that the differences in cyclin B1 protein and mRNA levels reported in Fig. 1 reflect the activities of c-Myc and p53 rather than differences in cell cycle profiles or viability.

Finally, to confirm that the increased levels of cyclin B1 transcripts described above were, at least in part, attributable to *de novo* mRNA transcription, we performed nuclear run-on assays in each of the various 32D or Rat1a cell lines. Cells maintained in log-phase growth at 38°C were placed at 32°C for 12 h. At the end of this time, the cells were then placed back at 38°C for 4 h to inactivate p53. Nuclei were then prepared for *in vitro* transcription as described previously (23), and the purified ^{32}P-labeled transcripts were hybridized to nitrocellulose filters containing affixed cyclin B1 cDNA or control plasmid DNAs. As seen in Fig. 2*A,* readily detectable cyclin B1 gene transcription was seen in control 32D-neo/hygro cells (*Row 1*), whereas both 32D-c-Myc/hygro and 32D-neo/tsp53 cells showed a 2–3-fold increased rate of transcription (*Rows 2* and *3*). The combination of c-Myc overexpression and p53 inactivation resulted in a ~10-fold enhancement in the rate of cyclin B1 gene transcription (*Row 4*). A similar pattern was seen in Rat1a cells (Fig. 2*B*) where c-Myc induction and p53 inactivation each resulted in a low level (~2–3-fold) enhancement of transcription (compare *Rows 2* and *3* with *Row 1*), whereas in combination, the rate of transcription was increased 8–10-fold (*Row 4*).

The Persistence of Cyclin B1 Transcripts. The short half-lives of both c-Myc protein and mRNA ($t_{1/2}$ ~30 min for each) have been well documented (35, 36). Thus, the persistence of cyclin B1 transcripts in the face of CHX blockade (Fig. 1, *D* and *E*) seemed initially somewhat paradoxical. On the other hand, several studies have demonstrated that c-Myc target gene transcripts often remain elevated long after c-Myc protein should have disappeared after the blockade of *de novo* protein synthesis (37–39). This disparity might be attributable to a prolonged half-life of the target gene's transcript and/or continued expression of the target gene after c-Myc no longer occupies the promoter. This would be consistent with c-Myc's role in promoting acetylation of target genes (40), as well as with the observation that transient expression of c-Myc can elicit long-term phenotypic effects (11). To address this issue, we studied the half-lives of MycER and cyclin B1 mRNAs. Rat11a-MycER/tsp53 cells were propagated continuously at 38°C and then treated for 12 h with 4-HT to activate c-Myc and induce high levels of cyclin B1. At the end of this time, fresh medium lacking 4-HT and containing 3 μg/ml Actinomycin D was added to inactive functional c-Myc and to block *de novo* RNA synthesis, respectively. Total RNA was then prepared at different times and examined by Northern blotting for the presence of MycER, cyclin B1, and GAPDH transcripts. As seen in Fig. 3, MycER transcripts, while clearly declining with time, did so more slowly than expected ($t_{1/2}$ ~1.5 h). In addition, cyclin B1 transcripts showed quite a long half-life (>6 h). From these studies, we conclude that the persistence of c-Myc-mediated induction of cyclin B1 transcripts, in the face of CHX blockade, is in large part a result of their long half-life, at least in Actinomycin D-treated cells.

Functional Interactions of c-Myc with the Cyclin B1 Promoter. The cyclin B1 promoter contains a single c-Myc type E-box element: CACGTG (33) 189-bp upstream of the start of transcription (Fig. 4*A*). To determine whether this represented an actual c-Myc binding site, we synthesized a ds oligonucleotide containing the E-box (WT sequence) and one with a mutant sequence (CACGTG → CTCGAG). Each was labeled to similar specific activities and used in EMSAs with recombinant c-Myc and Max(S) proteins (24). As seen in Fig. 4*B,* whereas neither c-Myc nor Max(S) alone bound the WT oligo-

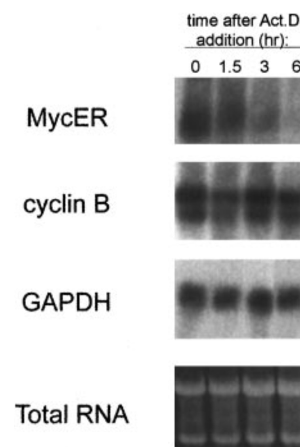

Fig. 3. Prolonged half-lives of MycER and cyclin B1 transcripts. Rat11a-MycER/tsp53 cells were propagated continuously at 38°C and then treated for 12 h with 4-HT to activate c-Myc and induce high levels of cyclin B. At the end of this time, fresh medium containing 3 μg/ml Actinomycin D was added to block *de novo* RNA synthesis. This fresh medium also lacked 4-HT, thus ensuring that c-Myc synthesis was blocked at both the transcriptional and pretranslational levels. Total RNA was then prepared at different times. Aliquots (5 μg) were subjected to Northern analysis for the presence of transcripts encoding MycER, cyclin B1, and GAPDH transcripts.

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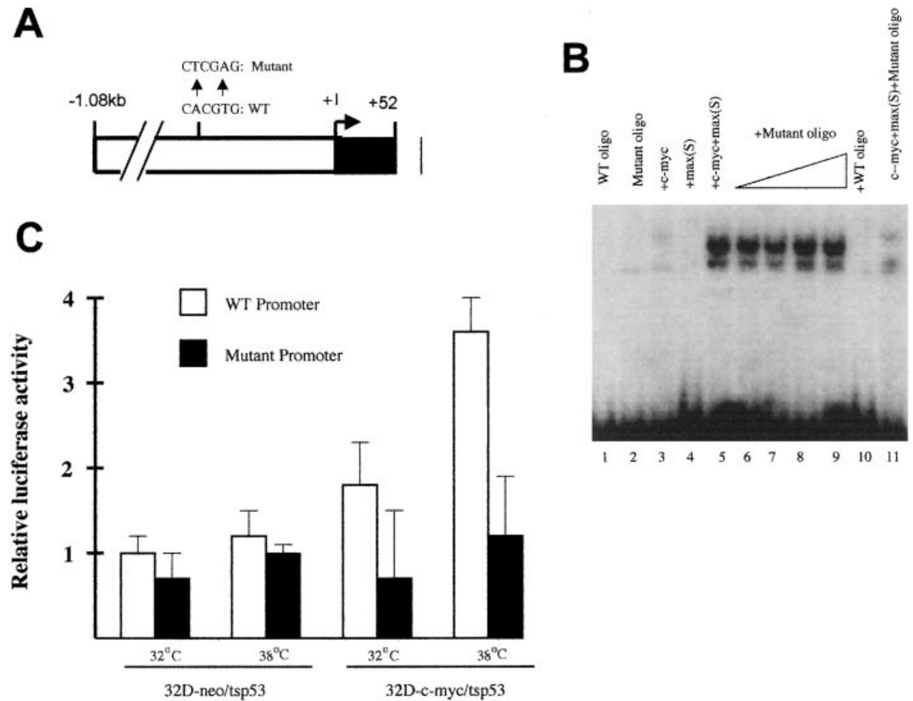

Fig. 4. The *cyclin B1* gene E-box binds c-Myc-Max heterodimers and confers c-Myc and p53 sensitivity. *A,* structure of the 1.08-kb cyclin B1 promoter. The c-Myc-type E-box element at position −189 (*WT*) is shown, as is the sequence of the mutant E-box used for EMSA and luciferase assays. The transcribed region is indicated in *black*. *B,* EMSAs, using ds oligonucleotides encompassing the WT or mutant E-box elements shown in *A,* were performed with the ^{32}P-labeled oligonucleotides alone (*Lanes 1* and *2*) or after incubation with ~20 ng each of affinity-purified, bacterially expressed c-Myc or Max(S) proteins (*Lanes 3–5*). In *Lanes 6–9,* the shifted complex obtained with the WT oligonucleotide was competed with 25, 50, 100, or 200 ng of unlabeled mutant ds oligonucleotide. In *Lane 10,* the same complex was competed with 25 ng of unlabeled WT ds oligonucleotide. c-Myc-Max(S) heterodimers did not bind the mutant ^{32}P-labeled oligonucleotide (*Lane 11*). *C,* luciferase assays in stably transfected 32D cells. The WT or mutant promoter fragments shown in Fig. 1*A* were cloned into the promotorless pGL2 luciferase expression vector and then stably expressed in 32D-neo or 32D-c-Myc cells along with the pCMVβgal-puro vector. Pooled, puromycin-resistant clones were then maintained at either 38°C or 32°C for 36 h before assaying for β-galactosidase and luciferase. The results shown are the results of quadruplicate determinations +/− 1 SE.

nucleotide (*Lanes 3* and *4*), a mixture of the two proteins (24) produced a shifted complex (*Lane 5*). Competition with excess unlabeled ds mutant oligonucleotide failed to diminish the signal (*Lanes 6–9*), whereas low concentrations of unlabeled WT oligonucleotide effectively competed for the labeled complex (*Lane 10*). Finally, no significant binding by c-Myc + Max(S) to the ^{32}P-labeled mutant ds oligonucleotide was seen (*Lane 11*). These experiments established that the cyclin B1 promoter E-box was able to specifically bind c-Myc-Max complexes *in vitro*.

To assess the c-Myc- and p53-dependent function of the cyclin B1 promoter *in vivo,* we constructed two luciferase expression vectors containing the 1.08-kb WT or mutant promoter fragments (Fig. 4*A*). Both vectors were introduced into 32D-neo/tsp53 or 32D-c-Myc/tsp53 cells. Stable clones were pooled and cultured at 32°C or 38°C for 36 h before performing luciferase assays. As shown in Fig. 4*C,* the WT cyclin B1 promoter in the context of c-Myc and mutant p53 expression was 3–4-fold more active than under any other conditions. In contrast, the promoter containing the mutant E-box was not up-regulated.

Constitutive Overexpression of Cyclin B1 Promotes Tetraploidy. Having determined that the cyclin B1 promoter is a direct transcriptional target for both c-Myc and p53, we next asked whether the constitutive overexpression of cyclin B1 could predispose cells to the development of tetraploidy. 32D-neo and 32D-c-Myc cells were therefore stably transfected with a human cyclin B1 expression plasmid or the control pAPuro vector. Western blots of cell lysates from the log phase or Nocodazole-treated pooled puromycin-resistant population showed high levels of cyclin B1 expression in the former two cell lines (Fig. 5). In contrast, control vector transfectants showed low levels of cyclin B1 that were indistinguishable from those seen in parental 32D cells (data not shown). Equivalent levels of the cdc2 catalytic subunit were detected in all four cell lines. Consistent with the elevated levels of cyclin B1 in cyclin B1 transfectants, these lines also contained elevated levels of cdc2 histone H1 kinase activity. Treatment with the mitotic spindle poison Nocodazole resulted in the expected increase in cyclin B1 and cdc2 kinase activities in control cells. In contrast, cells constitutively expressing cyclin B1 showed little cell cycle regulation of cyclin B1 and cdc2 kinase activity. These results indicate that 32D-neo and 32D-c-Myc cells could be successfully engineered to overexpress cyclin B1, thus resulting in the expected increase in cdc2 histone H1 kinase activity. Despite repeated attempts, we were unable to establish stable cyclin B1/tsp53 cell lines.

Characterization of the above four cell lines immediately after their derivation showed their cell cycle profiles to be identical and their DNA content to be uniformly diploid (Fig. 6*A*). Treatment of 32D-neo/puro cells with Nocodazole resulted in a >85% arrest in the G_2-M

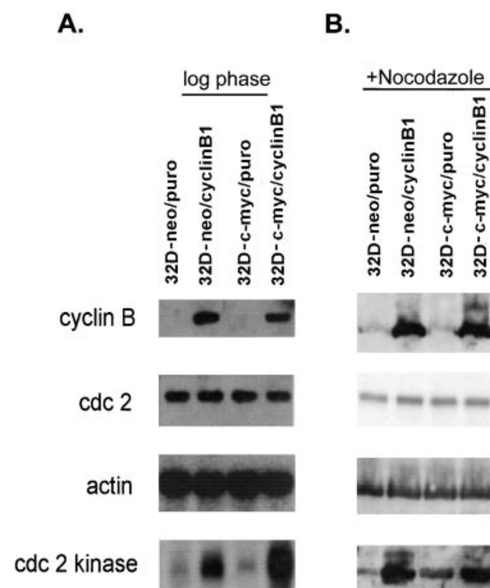

Fig. 5. Expression of cyclin B1, cdc2, and histone H1 kinase activity in 32D cells. 32D-neo or 32D-c-Myc cells were stably transfected with a cyclin B1 expression vector or the empty parental vector, both of which also encode Puromycin resistance. Puromycin-resistant clones in log phase growth (*A*) or after a 16-h exposure to Nocodazole (50 μg/ml) were pooled and subjected to Western blotting for the presence of cyclin B1 or cdc2 kinase. Histone H1 kinase activity was also assayed on cyclin B1 immunoprecipitates as described previously (12, 22).

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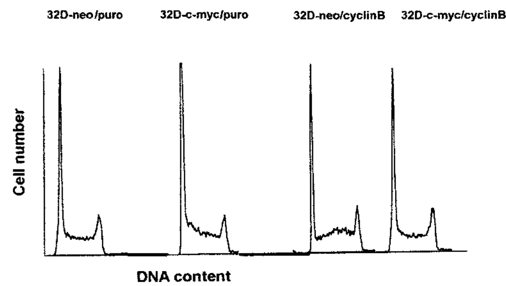

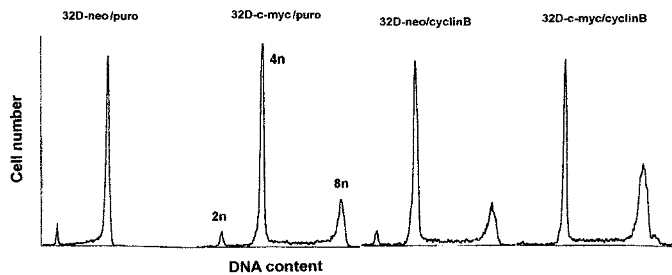

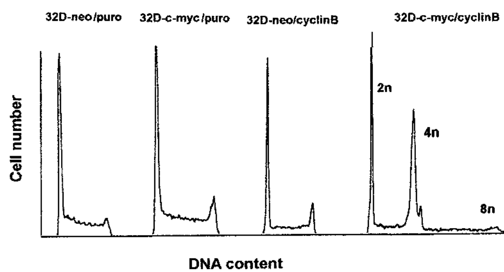

Fig. 6. DNA content of 32D cell lines. *A,* early passage log phase cell. *B,* the same cell lines exposed to Nocodazole (50 ng/ml × 16 h). *C,* log phase cells carried continuously in culture for 16–20 weeks.

phase of the cell cycle (Fig. 6*B* and Ref. 12). Nocodazole treatment of 32D-c-Myc/puro cells also promoted G_2-M arrest but also caused an uncoupling of mitosis and DNA synthesis. As a result, 10–15% of these cells became tetraploid. More importantly, 32D-neo/cyclin B1 cells also became tetraploid. Furthermore, the combination of c-Myc and cyclin B1 overexpression was additive and is reminiscent of our previous observations that c-Myc overexpression and p53 inactivation cooperate to induce tetraploidy (12). Similar results were observed in Rat1a cells (data not shown).

With continued passage, and in the absence of mitotic spindle inhibitors, 32D-c-Myc/tsp53 cells, but neither 32D-c-Myc nor 32D-tsp53 cells, gradually become tetraploid and eventually replace the diploid population (12). Thus, it was of interest that 32D-c-Myc/cyclin B1 cells also developed a small degree of tetraploidy on prolonged *in vitro* passage (Fig. 5*C*). However, this never comprised >15–20% of the entire population. This suggests that ectopic expression of cyclin B1 at least partly recapitulates the loss of p53 function with regard to the development of tetraploidy. Similar to what is seen in cells lacking functional p53, cyclin B1 overexpression alone not only promotes tetraploidy when the mitotic spindle is compromised but also cooperates with c-Myc to generate tetraploidy when the spindle apparatus is otherwise intact.

DISCUSSION

We demonstrated previously that overexpression of c-Myc or inactivation of p53 leads to the uncoupling of mitosis and DNA synthesis in cells treated with mitotic spindle inhibitors (12). The combination of c-Myc overexpression and p53 inactivation is not only additive in this regard but, in the absence of mitotic spindle inhibitors, also results in tetraploidy upon continuous *in vitro* passage. Increased cyclin B1 levels in these cells suggested that it is a target for both c-Myc and p53. The work presented here confirms this notion. We have shown that cyclin B1 itself, like c-Myc or loss of p53, is a sufficient stimulus to promote the development of tetraploidy under certain conditions. The lower level of tetraploidy that occurs in 32D-c-Myc/cyclin B1 cells, after prolonged *in vitro* passage, is less than that observed in 32D-c-Myc/tsp53 cells (12). This suggests that, in the latter case, additional p53 effectors may contribute to the generation of tetraploidy.

c-Myc regulates cyclins A, D1, and E (41, 42), as well as CDK4 (43). The c-Myc-mediated down-regulation of the cdk inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ (41, 44) may also play a role in promoting cell cycle progression (4). Significantly, a previous study, performed in Rat1a cells, failed to show regulation of cyclin B1 by c-Myc (45). In addition, our own and several subsequent cDNA microarray experiments have not identified cyclin B as a c-Myc-responsive gene (25, 39, 46–48). This is actually consistent with our current work showing that optimal induction of the cyclin B1 promoter by c-Myc only occurs when p53 is concurrently inactivated. In addition to cyclin B1, a number of other genes have been identified as negative p53 targets (16–18, 49–51).

It is noteworthy that, although the cyclin B1 promoter contains a functional c-Myc binding site (Fig. 4*A*), it contains no such consensus sites for p53. This has been pointed out previously by Taylor *et al.* (18), who showed that a p53-sensitive region of the promoter nonetheless resides within the region −123 to −287 relative to the start of transcription. In addition to the c-Myc E-box site, this region contains consensus binding sites for SP1 and E2F1, both of which have been shown to be regulated by p53 (52, 53). This suggests that, although the regulation of the *cyclin B1* gene by p53 does not require *de novo* protein synthesis, it is not direct in nature. Rather, it may be the result of p53's ability to modulate the activity and/or DNA binding of other preexisting transcription factors in the absence of ongoing protein synthesis.

It is also clear that the degree of regulation of the cyclin B1 promoter-luciferase reporter by c-Myc and p53 (~4-fold, Fig. 4*C*) is somewhat less than that observed when actual cyclin B1 transcript or protein levels are measured. This may indicate that additional c-Myc and p53-response elements exist elsewhere in the cyclin B1 promoter, that the luciferase reporters reside in chromosomal contexts different from that of the endogenous *cyclin B1* gene, and/or that luciferase mRNA and protein are subject to different types of posttranscriptional and posttranslational regulation than cyclin B1. Whatever the reasons for these differences, it is nevertheless clear that the relatively small segment of the cyclin B1 promoter used for our studies reflects in principal the inverse relationship that exists between c-Myc and p53 and their regulation of the endogenous *cyclin B1* gene.

Our observations, together with previous reports (7–12), suggest a model of how c-Myc, p53, and cyclin B1 play interdependent roles in maintaining genomic integrity (Fig. 7). In otherwise normal cells (Fig. 7*A*), DNA damage results in the p53-mediated inhibition of cell cycle progression in either G_1 or G_2-M (54, 55). In the latter case, this may be effected in part by the p53-mediated down-regulation of cyclin B1 (Refs. 16–18 and the current article). If not extensive, the acquired damage is repaired, and traversal through the cell cycle resumes. In

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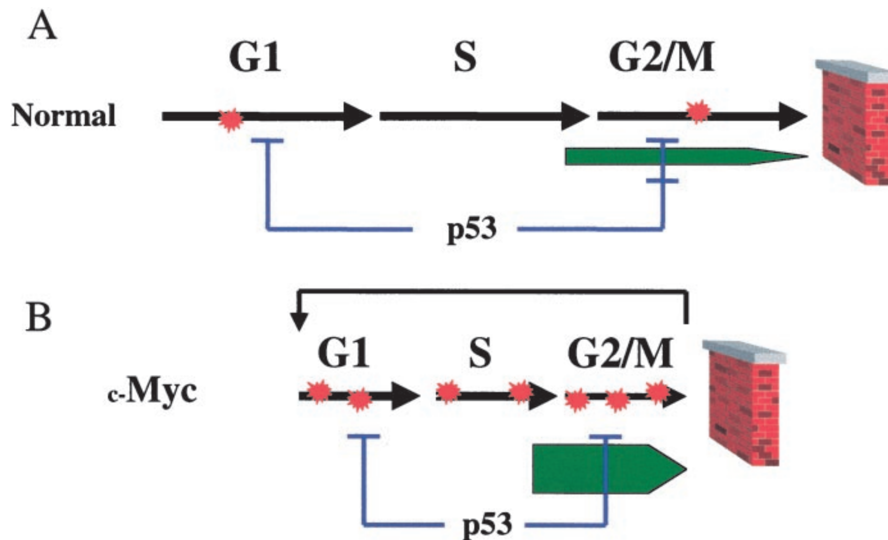

Fig. 7. Model for the role of c-Myc, p53, and cyclin B1 in the control of genomic instability. *A,* a "normal" cell in which endogenous c-Myc, cyclin B1, and p53 are under usual regulatory control and have not undergone any mutational alterations. G_1, S, and G_2-M, depicted by thick, *black arrows,* are of sufficient duration that most damage incurred during these times (*red*) is efficiently repaired during the period of p53-mediated cell cycle inhibition (*blue lines*). After DNA or mitotic spindle damage (*red*), p53 inhibits cyclin B1 (*green box*), thus helping to prevent the reinitiation of S phase in the absence of an intervening mitosis (*brick wall*). *B,* the shortened cell cycle that accompanies c-Myc overexpression. Such cells may acquire additional mutations as a direct result of c-Myc overexpression (7–11) and/or as an indirect result attributable to insufficient time to complete DNA repair processes (11). Such damaged cells would normally be eliminated by p53-dependent apoptosis (12) but would accumulate after loss of p53 surveillance function. The combination of p53 loss and c-Myc overexpression results in the synergistic up-regulation of cyclin B1, allowing cells to transit mitosis and reinitiate DNA synthesis after blockade of the mitotic spindle (*black line*). Enforced expression of cyclin B1 would have a similar effect (50) and, in addition, could help to overcome the G_2-M bock mediated by WT p53. Tetraploidy developing in the absence of extrinsic mitotic spindle inhibition as a result of concurrent c-Myc deregulation (Fig. 5*C*) might result from ongoing c-Myc-induced or spontaneously acquired mutations, as well as by the tendency of c-Myc to drive premature or inappropriate S phase entry.

such cells, mitotic spindle inhibition causes an accumulation of cells blocked in mitosis. In cells overexpressing c-Myc (Fig. 7*B*), G_1 and G_2-M are shortened, and S phase transition is accelerated (11, 56, 57), potentially allowing insufficient time for DNA repair and thus in the accumulation of a greater number of mutations. At least a portion of such damage, particularly that involving DNA amplification, may be the direct result of c-Myc overexpression (7–11). Loss of p53 would contribute to the accumulation of mutations by failing to eliminate the most extensively damaged cells (12). In addition, the combined effects of c-Myc overexpression and p53 loss would cause up-regulation of cyclin B1, thus providing additional mitotic thrust. In the presence of WT p53, the enforced expression of cyclin B1 might overcome the p53-imposed G_2-M block and again allow for the completion of mitosis (58, 59). Indeed, previous work has shown that, in some cases, enforced expression of cyclin B1 can override G_2-M arrest, suggesting that cyclin B1 is the rate-limiting step in the initiation of mitosis (59, 60). In any case, mitotic spindle inhibition only partially prevents the accumulation of mitotically blocked cells because of their tendency to reenter S phase. Cells that additionally overexpress c-Myc would be particularly prone to circumventing the mitotic spindle checkpoint block because of the tendency of c-Myc to initiate S phase (4). The failure of tetraploid 32D-c-Myc/cyclin B1 cells to become the predominant population after prolonged culture (Fig. 6*C* and *Panel 4*) may reflect the surveillance function of p53, which selects against maintenance of such a population. We would also emphasize that the effects of constitutive cyclin B1 expression on the promotion of tetraploidy need not be direct, *e.g.,* high levels of cyclin B1 might prevent cdc2 from associating with other cyclins, most notably cyclin A, during S phase. The consequences of such a potential cyclin-cdk imbalance are currently unknown.

It is of interest that the cyclin B1 E-box at position −189 (Fig. 4) has been described previously as a binding site for the bHLH-ZIP transcription factor USF (33). USF levels are cell cycle regulated, reaching their peak during G_2-M, the time when cyclin B1 gene expression is also maximal. Endogenous c-Myc levels, on the other hand, are largely invariant throughout the cell cycle (61). This suggests an indirect interaction between USF and c-Myc, perhaps involving the displacement of one protein for another (62). Potential coregulation by c-Myc and USF has also been described for *cad,* another c-Myc target gene (63). Finally, other indirect positive effects on the cyclin B1 promoter might result from the previously described c-Myc-induced up-regulation of cyclins A, D1, and E (64).

The codependence of c-Myc and p53 has not been described previously for other c-Myc targets. Cyclin B1 thus represents the prototype of a new class of c-Myc targets, the positive regulation by c-Myc of which is counterbalanced by a potent tumor suppressor such as p53. The central role of cyclin B1 in the maintenance of genomic integrity is underscored by the consequences of its deregulated expression. It will be of interest to determine whether other c-Myc-regulated target genes are subject to similar negative control by p53 or other tumor suppressors.

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