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Constitutive expression of B-*myb* can bypass p53-induced Waf1/Cip1-mediated G_1 arrest

(oncogene/suppressor gene/cell cycle)

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ABSTRACT **Overexpression of wild-type p53 protein has been shown to induce arrest in the G_1 stage of the cell cycle and to transactivate expression of the gene that encodes the 21-kDa Waf1/Cip1 protein, a potent inhibitor of cyclin-dependent kinase activity. p53-dependent G_1 arrest is accompanied by decreased expression of the B-*myb* gene, a relative of the c-*myb* cellular oncogene. In this study we show that B-*myb* expression is required for cells to progress from G_1 into S phase and that high levels of ectopic B-*myb* expression uncoupled from cell cycle regulation rescues cells from p53-induced G_1 arrest even in the presence of Waf1/Cip1 transactivation and inhibition of cyclin E/Cdk2 kinase activity. Cotransfection experiments with p53 expression plasmids and expression plasmids encoding in-frame deletion mutations in B-*myb* coding sequences indicate that the DNA-binding domain of the B-Myb protein is required for this activity. These results provide evidence of a bypass of p53-induced Waf1/Cip1-mediated cell cycle regulatory pathways by a member of the *myb* oncogene family.**

The p53 tumor-suppressor gene has been identified as a participant in cell cycle control, DNA synthesis and repair, maintenance of genomic stability, cellular differentiation, and programmed cell death (1–3). The p53 protein has been shown to be a modulator of transcription which can exhibit both positive and negative effects on gene expression (4). The functional versatility of p53 appears to depend on cellular context, interactions with specific DNA sequences, interactions with other proteins, and changes in p53 protein conformation (3–5).

We have shown that overexpression of wild-type p53 in a human glioblastoma tumor cell line carrying a hormone-inducible p53 cDNA transgene arrests cell cycle progression at or near a restriction point controlling the G_1/S-phase transition (6), now referred to as a G_1 checkpoint (7–10). G_1 arrest in this model was accompanied by a marked decrease in expression of a number of endogenous genes fundamental for cell cycle progression and DNA replication, including B-*myb* and those encoding proliferating-cell nuclear antigen and DNA polymerase α (6). A potential explanation for the molecular basis of G_1 arrest in this model was provided by the demonstration that p53 transcriptionally activates expression of a gene that encodes a 21-kDa protein, Waf1/Cip1 (11, 12), that is a potent inhibitor of cyclin-dependent kinase (Cdk) activity (13).

The observation that B-*myb* expression is dramatically decreased in G_1-arrested cells was of particular interest for several reasons. The B-*myb* gene was first identified by homology to the c-*myb* protooncogene (14). The protein product of the B-*myb* gene is a sequence-specific DNA-binding transcription factor (15, 16). B-*myb* mRNA levels are low or undetectable in quiescent cells; however, following mitogenic stimulation, B-*myb* mRNA increases sharply at the G_1/S-phase transition (17, 18). Accordingly, B-*myb* is thought to play an important role in regulating the expression of late G_1/S-phase genes directly involved in DNA replication (19–21). In the present study we sought to determine the effect of constitutive expression of B-*myb* on p53-induced G_1 arrest mediated by Waf1/Cip1. Evidence is presented indicating that constitutive B-*myb* expression rescues cells from p53-induced G_1 arrest mediated by the Waf1/Cip1 gene.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The GM47.23 (GM) cell line was cultured as described by Mercer *et al.* (22). The human osteosarcoma tumor cell line Saos-2 (American Type Culture Collection HTB 85) was cultured as described by Fiscella *et al.* (23). The GM/MYB sublines were derived from the parental GM cell line by transfection with expression plasmid pCMV-B-myb (22).

B-*myb* Oligonucleotides and Microinjection. Unmodified oligonucleotides were synthesized on an automated solid-phase DNA synthesizer (Applied Biosystems model 392) by standard phosphoramidite chemistry. Antisense oligonucleotides were all 18-mers and had the following 5′-to-3′ sequences: B-MYB1, GCAGCGCGTCCGCCGAGA; B-MYB2, CAGCTCATCCAGATCCTC; B-MYB3 mismatch, GCACCGCCTCGGCGGAGA. Stock solutions of antisense oligomers were prepared in 10 mM Tris·HCl (pH 7.5). GM cells were first growth-arrested by a combination of high cell density and serum deprivation (22). Cells were stimulated to reenter the cell cycle by replating them at a 1:4 split ratio on glass coverslips imprinted with areas for microinjection as described (24).

B-*myb* Expression Plasmids. The human B-*myb* cDNA insert cloned in the SK plasmid vector (Stratagene) pSK-B-myb4 (21) was excised from vector sequences by use of the *Bam*HI restriction enzyme and recloned into the *Bam*HI restriction site of the pCMV-Neo-Bam expression vector (25). B-*myb* deletion mutants were produced from the B-*myb* cDNA insert of pSK-B-myb4. Mutant 1 was generated by deletion of the *Tth*111I–*Xho* I DNA fragment (nt 368–743; nucleotide numbers are as described in ref. 26). To generate mutant 7, an *Xho* I restriction site was generated by oligonucleotide site-directed mutagenesis and the 192-bp *Xho* I fragment (nt 744–935) was deleted. The deletion-mutant B-*myb* cDNA inserts were excised from the SK plasmid vector by *Bam*HI digestion and recloned into the *Bam*HI site of the pCMV-Neo-Bam expression vector. The mutant 1 expression plasmid was designated pCMV-MUT-1 and the

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Abbreviation: WTp53, wild-type p53.
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mutant 7 plasmid was designated pCMV-MUT-7. Deletions were verified by DNA sequence analysis of the B-*myb* cDNA inserts.

Transient-Transfection Experiments. Exponentially growing Saos-2 cells were transfected with plasmid DNA (27). The total amount of plasmid DNA in each suspension transfection mixture was 40 μg per 2×10^6 cells. Transfected cells were evaluated for p53 expression 24 hr posttransfection by immunocytochemical staining using monoclonal antibody PAb 1801 (Oncogene Science, Ab-2) followed by a secondary antibody conjugated with horseradish peroxidase. The fraction of labeled cells was determined by continuous labeling with [*methyl*-^{3}H]thymidine (0.2 μCi/ml; 1 μCi = 37 kBq) for 24 hr, followed by standard autoradiography and light microscopic counting.

Establishment of GM/MYB Sublines. GM/MYB sublines were established from the parental GM cell line (22) by transfection with the pCMV-B-MYB expression vector. Individual clones were selected and expanded in growth medium containing G418 (GIBCO) at 800 μg/ml (23). G418-resistant clones were examined for induction of wild-type p53 (WTp53) protein at 12–18 hr after treatment with 1 μM dexamethasone (Sigma), by immunofluorescence staining using WTp53-specific monoclonal antibody PAb 1620 (Oncogene Science, Ab-5) followed by a secondary antibody conjugated with fluorescein isothiocyanate. GM/MYB clonal sublines designated MYB-2, MYB-6, MYB-8, and MYB-10 were established.

Northern Blot Analysis and Hybridization Probes. Total RNA was extracted (28) and Northern blots were prepared (29). Plasmid DNA was isolated and purified by column chromatography (Qiagen, Chatsworth, CA). Hybridization probes were the 1.8-kb *Xba* I cDNA fragment of plasmid p53H (22), the 2.0-kb *Bam*HI cDNA fragment of plasmid pSK-B-myb4 (21), and the 2.1-kb *Not* I cDNA fragment of plasmid pLZ-WAF1 (11). The cDNA inserts were purified from vector sequences by agarose gel electrophoresis and labeled to high specific activity with [α-^{32}P]dCTP (30).

Kinase Assays and Western Blot Analysis. Total cell lysates were prepared for kinase assays or Western blotting as described (31). Cyclin E was immunoprecipitated from cell lysates with an anti-cyclin E polyclonal antibody (Upstate Biotechnology) and immune-complex kinase activity assayed against histone H1 (31). For Western blotting, samples (50 μg) of total cell protein was electrophoresed in SDS/10% polyacrylamide gels and the separated proteins were transferred to poly(vinylidene difluoride) membranes in a semidry blotting apparatus (Hoefer). Blots were sequentially probed with antibodies to cyclin E and Cdk2 (Upstate Biotechnology), cyclin A (kindly provided by Giulio Draetta, Mitotix, Cambridge, MA), and Cdc2 (ICN). Primary antibody interaction was detected with horseradish peroxidase-conjugated second antibodies by enhanced chemiluminescence (ECL) procedures (Amersham).

RESULTS

Effects of B-*myb* Antisense Oligonucleotides on G_1/S Progression. We sought to determine whether B-*myb* expression was required for the GM cell line to progress from G_1 into S phase. The effect of microinjecting various concentrations of B-*myb* oligomers is shown in Fig. 1. The B-MYB1 and B-MYB2 antisense oligomers were capable of inhibiting G_1/S-phase progression at each concentration tested relative to the B-MYB mismatch control oligomer. However, we were unable to quantitate B-Myb protein levels in these cells because antibodies to B-Myb are not available.

Constitutive B-*myb* Expression Rescues Cells from WTp53-Induced G_1 Arrest. We next sought to determine whether constitutive high levels of B-*myb* expression could rescue cells from WTp53-induced Waf1/Cip1-mediated G_1 arrest. Four independent clones, MYB-2, MYB-6, MYB-8, and MYB-10, were identified which retained inducible expression of the WTp53 protein (data not shown). We first examined the effect of inducing WTp53 protein expression on G_1 progression into S phase. Fig. 2 shows the results of a typical experiment. Induction of WTp53 protein in the MYB-2, MYB-6, and MYB-10 cell lines failed to inhibit G_1 progression into S phase. In contrast, and as expected, cell cycle progression was inhibited in the parental GM cell line induced to express WTp53 protein (6, 11, 22). Inhibition was also observed in MYB-8 cells, indicating that they behave phenotypically like parental GM cells.

Gene Expression in MYB Sublines. Northern blot analysis was performed to examine p53, B-*myb*, and Waf1 gene expression in the GM/MYB cell lines. A representative composite Northern blot of RNA prepared from the MYB-2 cell line is shown in Fig. 3. The endogenous mutant p53 mRNA transcript of 2.8 kb is constitutively expressed in MYB-2 cells as in parental GM cells (6, 22). The WTp53 transgene mRNA transcript of 2.0 kb is expressed only in induced cells. The endogenous B-*myb* mRNA transcript of 2.7 kb and a smaller, 2.0- to 2.3-kb mRNA transcript of the

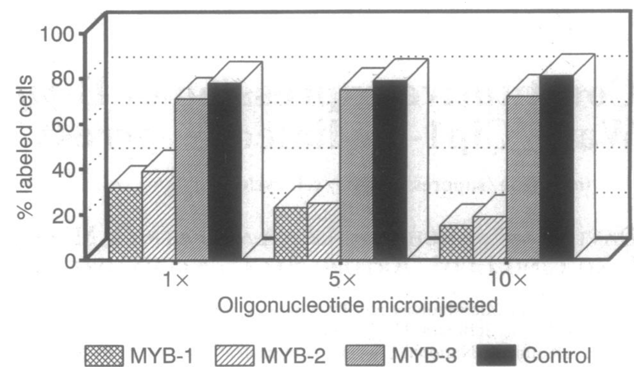

FIG. 1. Effect of B-*myb* oligonucleotides on cell cycle progression. GM cells were microinjected with B-*myb* oligomers MYB-1, MYB-2, or MYB-3 mismatch. Immediately following microinjection, [^{3}H]thymidine (0.2 μCi/ml) was added to the cell cultures. Cells were labeled continuously for 24 hr and then fixed and processed for autoradiography. The percentage of labeled cells was determined by light-microscopic counting. Approximately 500 cells were microinjected with oligonucleotides at concentrations of 0.1 μg/ul (1×), 0.5 μg/ul (5×), and 1.0 μg/ul (10×). Non-microinjected cells grown on the same coverslip served as control background cells.

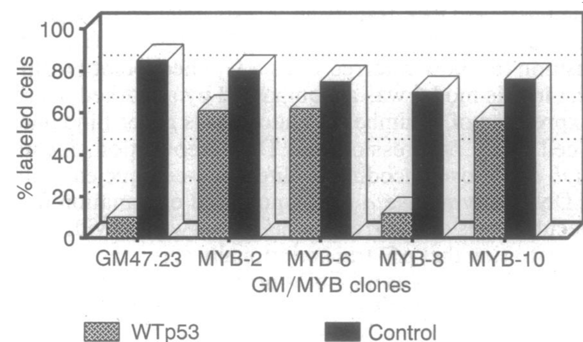

FIG. 2. Effect of constitutive expression of B-*myb* on WTp53-induced G_1 arrest of GM/MYB sublines. MYB-2, MYB-6, MYB-8, and MYB-10 sublines were growth arrested in G_0 and stimulated to reenter the cell cycle without (black bars) and with (stippled bars) induction of WTp53 protein by dexamethasone treatment. At the time of stimulation [^{3}H]thymidine (0.2 μCi/ml) was added and cultures were labeled for 24 hr. The cells were then fixed and processed for autoradiography and the fraction of labeled cells was determined by light microscopy.

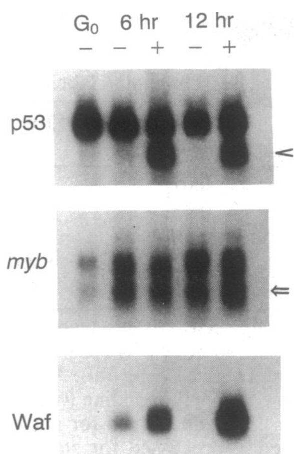

FIG. 3. Composite Northern blot analysis of mRNA expression in MYB-2 cells. Total RNA was isolated from MYB-2 cells growth arrested (G_0) or stimulated to reenter the cell cycle without (–) and with (+) induction of the WTp53 transgene at the times indicated. RNA (10 μg per lane) was size fractionated by agarose gel electrophoresis and Northern blots were prepared. The blots were sequentially hybridized with ^{32}P-labeled p53, B-*myb* and Wafl cDNA probes. The arrowhead shows the position of the mRNA transcript of the inducible WTp53 transgene. The arrow shows the position of the mRNA transcript of the constitutively expressed B-*myb* transgene.

B-*myb* transgene are expressed constitutively in both growth-arrested and stimulated cells, independent of WTp53 induction. On the contrary, the endogenous Wafl mRNA transcript of 2.1 kb is expressed at high levels only in cells induced to express WTp53 protein, as previously reported for parental GM cells (11). Identical results were found in Northern blots prepared from the MYB-6 and MYB-10 sublines (data not shown). Thus, WTp53 transgene and endogenous Wafl gene expression in these three GM/MYB sublines is identical to that observed in parental GM cells (11). In contrast, as shown in the composite Northern blot of Fig. 4, the B-*myb* transgene mRNA transcript is not expressed in the MYB-8 subline and, unlike the constitutive expression pattern of the endogenous B-*myb* mRNA observed in the other three GM/MYB sublines, endogenous B-*myb* mRNA in MYB-8 cells is not expressed in growth-arrested cells or stimulated cells induced to express WTp53 protein. This pattern of endogenous B-*myb* expression in the MYB-8 cell line is identical to that observed in parental GM cells (6).

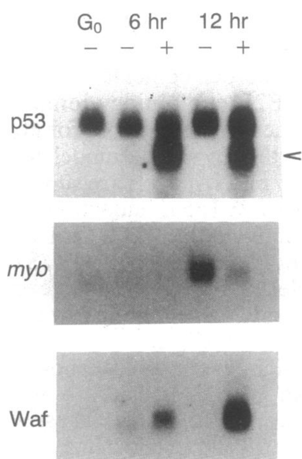

FIG. 4. Composite Northern blot analysis of mRNA expression in MYB-8 cells. MYB-8 cells were treated as described in the legend of Fig. 3. Total RNA was isolated and Northern blots were hybridized with ^{32}P-labeled p53, B-*myb*, and Wafl cDNA probes.

Rescue of Cells Does Not Require Activation of Cyclin E/Cdk2. G_1 arrest induced by WTp53 involves Wafl/Cip1-mediated inhibition of the G_1 cyclin-dependent kinases (32, 33). We therefore investigated the possibility that B-*myb* might prevent WTp53 from inducing a G_1 arrest by blocking Wafl/Cip1-mediated inhibition of cyclin E/Cdk2 activity. We chose to concentrate our efforts on the activity of the cyclin E/Cdk2 complex, since this G_1 cyclin-dependent kinase plays a critical role in promoting the G_1/S-phase transition (34). The parental GM cell line and the MYB-2 cell line were synchronized in G_0 and then stimulated to reenter the cell cycle in the presence or absence of WTp53 induction. G_0-arrested cells exhibited low basal levels of cyclin E kinase activity which increased >20-fold as cells reached the G_1/S-phase border (Fig. 5). Induction of WTp53 in either GM or MYB-2 cells prevented activation of cyclin E/Cdk2 complexes, demonstrating that B-*myb* expression does not block the ability of WTp53 to inhibit this G_1 cyclin-dependent kinase. Western blotting revealed that the basal levels of cyclin E and Cdk2 were relatively similar in GM and MYB-2 cells. Cdc2 protein levels in the growth-arrested MYB-2 cells were, however, relatively higher than in GM cells (Fig. 5). This result is consistent with the reported increase of Cdc2 mRNA in cells overexpressing B-*myb* (21). We then measured the level of the cyclin A protein as a marker for cells committed to S phase: cyclin A protein synthesis commences in late G_1 (or early S phase) and protein levels continue to accumulate as cells progress through S phase (35). Consistent with this notion, cyclin A protein levels were relatively low in GM and MYB-2 cells arrested in G_0 and increased as the control cultures entered S phase (Fig. 5). Induction of WTp53 suppressed the accumulation of cyclin A and also the Cdc2 protein in GM cells, but to a lesser degree in MYB-2 cells.

B-Myb DNA-Binding Domain Is Required for Rescue. Previous studies have shown that transfection of pCMV-WTp53, a WTp53 cDNA expression plasmid under transcriptional control of the strong constitutive cytomegalovirus promoter, inhibits the growth of Saos-2 cells (7, 23), which are p53-null cells and do not express endogenous p53 mRNA or protein (7, 23, 36). We used this assay to investigate which domains of the B-Myb protein are required to rescue cells from WTp53-induced growth arrest. Control experiments were first performed in which the pCMV-Neo-Bam vector was replaced with a pCMV-SV40 plasmid expressing the simian virus 40 large tumor (T) antigen. The pCMV-SV40-T expression plasmid efficiently rescued cells from WTp53-induced growth arrest as indicated by the increase in the percentage of p53-positive labeled cells shown in Table 1. Experiments were then performed in which cells were cotransfected with the pCMV-B-MYB, pCMV-MUT-1, or pCMV-MUT-7 expression plasmid and the pCMV-WTp53 expression plasmid at a ratio of 10:1. As indicated in Table 1, cotransfection with pCMV-B-MYB and pCMV-WTp53 resulted in an efficient rescue of p53-positive cells from WTp53-induced growth arrest. On the contrary, pCMV-MUT-1, which lacks the sequence encoding the DNA-binding domain, completely failed to rescue cells. Cotransfection with pCMV-MUT-7 was able to rescue p53-positive cells from WTp53-induced growth to some extent; however, repeated experiments consistently revealed that pCMV-MUT-7 was less efficient than pCMV-B-MYB.

DISCUSSION

Induction of WTp53 protein in GM cells transactivates the Wafl/Cip1 gene (11). Recent studies have shown that Wafl/Cip1 mRNA dramatically increases in cells expressing WTp53 that arrest in G_1 following DNA damage. The rise in

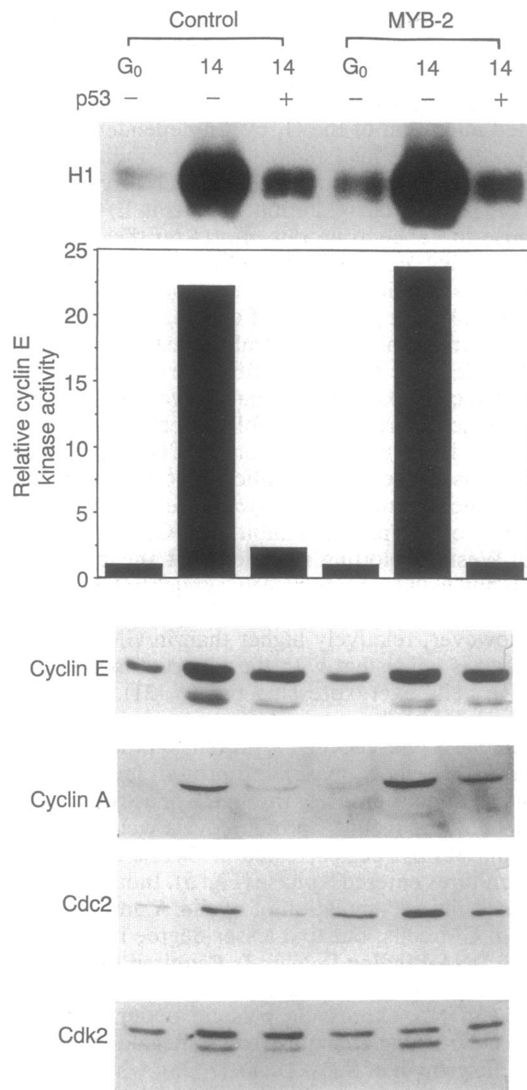

FIG. 5. Effect of WTp53 induction and constitutive high-level expression of B-*myb* on G_1/S-phase cyclin-dependent kinases. GM and MYB-2 cells were growth arrested in G_0 and then stimulated to reenter the cell cycle without (−) or with (+) WTp53 induction. To trap cells at the G_1/S-phase border and maximize cyclin E/Cdk2 kinase activity, aphidicolin (1 μM) was included in the medium used to stimulate entry of cells into the cycle. Total cell lysates were prepared at 0 and 14 hr following release from G_0 (the G_1/S-phase transition in these cells occurs 10–12 hr after release from G_0). (*Top*) Cyclin E/Cdk2 kinase activity was assayed by the amount of ^{32}P incorporated into histone H1 substrate. Quantitation was performed with a Betascope analyzer (Betagen, Waltham, MA) following gel electrophoresis of the reaction mixture. (*Middle*) Amount of kinase activity in each sample relative to the amount of kinase activity in G_0-arrested cells. (*Bottom*) Western blotting was performed to ascertain the levels of cyclins A and E, Cdc2, and Cdk2 in the cells.

Waf1/Cip1 mRNA levels parallels the levels of p21 protein found in cyclin E/Cdk2 complexes which exhibit decreased protein kinase activity (33). Our studies show that constitutive high-level expression of B-*myb* can bypass this p53-dependent G_1 arrest mechanism despite the induction of Waf1/Cip1 and inhibition of cyclin E/Cdk2 kinase activity. These findings lead us to conclude that B-Myb has the capacity to reduce the normal requirement of cells for cyclin E/Cdk2 kinase activity to enter S phase. MYB-2 cells appeared to commit to S phase despite having lower levels of cyclin A. G_1/S-phase progression in MYB-2 cells therefore appears to rely less on the level of cyclin E/Cdk2 kinase activity and possibly cyclin A protein than parental cells.

Table 1. Effect of B-*myb*-expressing plasmids on WTp53-induced growth arrest in Saos-2 cells

Effector plasmid	[^{3}H]Thymidine labeling index: p53-positive cells	[^{3}H]Thymidine labeling index: p53-negative cells	Rescue, %
pCMV-Neo-Bam	0.07	0.60	12
pCMV-SV40-T	0.45	0.63	71
pCMV-B-MYB	0.40	0.58	69
pCMV-MUT-1	0.05	0.64	8
pCMV-MUT-7	0.22	0.60	38

Exponentially growing Saos-2 cells were cotransfected with the WTp53 expression plasmid pCMV-WTp53 (4 μg/ml) and each of the effector plasmids listed above (36 μg/ml). At 24 hr after transfection the cells were exposed to [^{3}H]thymidine (0.2 μCi/ml) for 18–20 hr. The cells were then fixed and stained for nuclear p53 protein. The cells were subsequently processed for standard autoradiography. The transfection efficiency as determined by immunocytochemical staining for p53 protein ranged from 20% to 30%. The efficiency of rescue was calculated from the labeling index (LI) as follows: % rescue = (LI of p53-positive cells/LI of p53-negative cells) × 10^2.

The B-Myb DNA-binding domain and, to a lesser extent, the acidic transactivating domain were required to rescue cells from WTp53-induced G_1 arrest (Table 1). This suggests that the S-phase-promoting activity of B-Myb may involve transcriptional activation of late G_1 and/or S-phase genes that act downstream of the WTp53 G_1 checkpoint. In this way B-Myb might simply bypass WTp53-dependent G_1 arrest and promote entry into S phase.

The endogenous B-*myb* transcript is constitutively expressed in all three GM/MYB sublines (Fig. 4). This demonstrates that the endogenous B-*myb* promoter is active even in the presence of Waf1/Cip1 induction in these cells. This observation raises the possibility that constitutive high-level expression of the B-*myb* transgene product can subvert, either directly or indirectly, the repressing activity of factors regulating endogenous B-*myb* transcription. The human B-*myb* promoter has not been analyzed in detail. However, the mouse B-*myb* promoter shows a complex pattern of repression and derepression mediated, at least in part, through binding sites for E2F transcription factor-containing complexes (37). Multiprotein complexes containing E2F have been observed to interact with E2F binding sites in the B-*myb* promoter, and the composition of these complexes changes as cells progress from G_0/early G_1 into S phase. Lam and Watson (37) have suggested that B-*myb* promoter repression in G_0 could be mediated through the association of E2F with the retinoblastoma (RB) protein and/or related proteins. Derepression of B-*myb* transcription which occurs at the G_1/S-phase border coincides with alterations in the composition of E2F-containing complexes. If cyclin-dependent kinase activity is required for derepression of the B-*myb* promoter, then a simple model might explain why endogenous B-*myb* mRNA levels are suppressed in GM cells induced to express WTp53: induction of WTp53 would transcriptionally activate the Waf1/Cip1 gene, which would inhibit the derepressing activity of the G_1/S cyclin-dependent kinases. This model provides a reasonable explanation for the decreased B-*myb* mRNA levels observed in parental cells induced to express WTp53. It does not, however, explain how constitutive high-level expression of a B-*myb* transgene rescues cells from WTp53-mediated repression of endogenous B-*myb* transcription or the G_1 arrest. One possibility is that the B-*myb* transgene product may positively regulate endogenous B-*myb* gene transcription. In support of this possibility, positive autoregulation of the human c-*myb* promoter by the c-Myb protein acting through Myb-binding sites has been reported (38). Alternatively, B-Myb could act indirectly by activating genes whose products relieve re-

straints placed on the E2F transcription factor by RB and/or RB-related proteins such as p107. This would result in a greater proportion of free E2F to "turn-on" S-phase genes. Obviously additional studies will be required to elucidate more fully the mechanism by which constitutive expression of B-*myb* rescues cells from WTp53-induced G_1 arrest.

The human papillomavirus 16 E7 oncoprotein has recently been shown to disrupt p107/E2F complexes, resulting in release of active E2F and derepression of B-*myb* gene transcription (39). E7-transfected cells exhibit deregulated B-*myb* transcription which results in constitutive high-level expression, even during early G_1, when B-*myb* is normally transcriptionally silenced. Considering that B-*myb* plays an important role in cell cycle progression (19), its deregulation by E7 might contribute to the mitogenic activity of this viral oncoprotein. Based on the present study and the report by Lam *et al.* (39), one might expect that E7-transfected cells would also lack the ability to arrest in G_1 following induction of WTp53. There is some recent support for this notion: Slebos *et al.* (40) have reported that E7 can disrupt the p53-dependent G_1 cell cycle checkpoint activated by DNA damage. Thus, constitutive overexpression of B-*myb*, or deregulation of B-*myb* transcription through expression of E7, appears to enable cells to override WTp53-induced G_1 arrest through the Waf1/Cip1 pathway. Functional assessment of the G_1 checkpoint and molecular analysis of B-*myb* expression in tumor cells might alert one to deregulation of this G_1 checkpoint in cells harboring a functionally intact p53-control system. Furthermore, therapeutic strategies could be devised to exploit such defects. One approach might entail activating the G_1 checkpoint and then selectively killing cells that progress "unchecked" into S phase. Cells which failed to arrest in G_1 would be vulnerable to such manipulation, whereas normal cells that remained stably arrested in G_1 would be spared from toxicity.

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