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B-*myb* Promotes S Phase and Is a Downstream Target of the Negative Regulator p107 in Human Cells*

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The retinoblastoma protein family has been implicated in growth control and modulation of the activity of genes involved in cell proliferation, such as B-*myb*. Recent evidence indicates that the product of the B-*myb* gene is necessary for the growth and survival of several human and murine cell lines. Upon overexpression, B-*myb* induces deregulated cell growth of certain cell lines. Here we show that B-*myb* overexpression is able to induce DNA synthesis in p107 growth-arrested human osteosarcoma cells (SAOS2). p107 might exert its growth-suppressive activity by regulating B-*myb* gene transcription. Indeed, p107 down-modulated B-*myb* promoter activity and drastically decreased E2F-mediated transactivation. Finally, B-*myb* was able to stimulate DNA synthesis of both stably and transiently transfected human glioblastoma cells (T98G). Altogether, these data provide definitive evidence that the human B-*myb* protein is involved in growth control of human cells, and that p107 has a significant role in regulating B-*myb* gene activity.

B-*myb* has been cloned by virtue of its homology with the c-*myb* protooncogene (1) and its structure indicates that it may be a transcriptional activator. However, conflicting data have hampered a definitive evaluation of B-*myb* function(s). Some investigators have provided evidence of transcriptional activation of promoters containing Myb-responsive elements by B-*myb* (2, 3), whereas others showed that B-*myb* is unable to transactivate these promoters, acting rather as a repressor of c-*myb* transcriptional activity (4, 5). A recent report has established that B-*myb* transcriptional activity is highly dependent on the cell type (6), thus explaining the sharp discrepancies in B-*myb*-mediated biological effects observed in different systems. It has been clearly shown, however, that B-*myb* is required for the proliferation of murine fibroblasts, human glioblastoma, human leukemic cells lines, and human neuroblastoma cells (7–10) and that deregulated expression of B-*myb* induces abnormal cell growth and activation of genes such as cyclin D1 and *cdc2* in rodent fibroblasts (7). Consistent with the notion that B-*myb* may be a regulator of the cell cycle, B-*myb* expression has been correlated with cell proliferation in different systems and is induced at the G_1/S border of the cell cycle (11–14). Furthermore, ectopic expression the HPV16 E7 transforming protein induces up-regulation of B-*myb* expression, whereas growth suppression agents like TGF-β or the p53 protein cause down-regulation of B-*myb* mRNA levels (15–17). In a recent study it was provided evidence that B-*myb* can bypass p53-induced Waf1/Cip1-mediated G_1 arrest (8). p21Waf1/Cip1 can inhibit G_1 cyclin protein kinase and the phosphorylation of pRb[1] (18), suggesting that B-*myb* may overcome retinoblastoma-associated functions.

The retinoblastoma family comprises three members, pRb, p107, and p130, which share structural and functional characteristics. These proteins all induce growth arrest when overexpressed in certain cell lines and are able to bind the viral transforming protein E1A and the E2F family of transcription factors (19–25). It is notable that p107, but not pRb, can inhibit DNA synthesis of C33A cervical carcinoma cells (25); furthermore, E2F-1 can rescue pRb-mediated growth arrest of SAOS2 osteosarcoma cells, whereas it is ineffective in bypassing a p107 block (25, 26). Recent evidence indicates that p107 and pRb may associate *in vivo* with specific E2F proteins (we will use the term E2F to indicate all the possible members of the family); pRb has been found associated in cell extracts with E2F1, E2F2, and E2F3 (27), whereas p107 seems to bind specifically E2F4 (19, 22). However, when E2F1 and p107 are transiently transfected an interaction can be observed (19), suggesting that weak binding between p107 and E2F1 may also exist *in vivo* but can be detected only by overexpression. There is mounting evidence that E2F and the associated pRb family proteins are involved in cell cycle regulation of B-*myb* transcription (14, 15, 28). Thus, a conserved E2F site is located in the human and mouse B-*myb* promoters within a region specifying the multiple 5′ termini of the mRNA. It has been proposed that specific E2F-p107 and E2F-p130 complexes repress transcription in G_0/G_1 through binding to this site; induction of B-*myb* transcription at the G_1/S boundary, which appears to arise predominantly by a derepression mechanism, correlates with modification of these complexes by cyclin A/Cdk2 association (14, 15). Further evidence that that p107 is responsible for transcription repression in murine NIH 3T3 fibroblasts was provided by the finding that induction of B-*myb* transcription by HPV E7 protein mutants correlated with their ability to bind p107 rather than pRb (15). These findings indicate that B-*myb* may be a downstream target of p107 and, as such, suggest a mechanism by which this protein may act as growth suppressor. To provide

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[1] The abbreviations used are: pRb, the product of the retinoblastoma gene; CMV, cytomegalovirus; HPV, human papilloma virus.

further evidence for this hypothesis, we have investigated the functional relationship among B-*myb*, p107, and pRb in human cells.

MATERIALS AND METHODS

Cell Lines—The human cell lines SAOS-2, T98G, GM47, and B-*myb* derivatives were routinely maintained and transfected as described previously (7, 8).

Plasmids—The pCMV-B-*myb* and pCMV-B-*myb* mutant and the pCMV-based p107, pRb, E2F1, E2F4, DP1, and CD20 plasmids were all described. (8, 22, 24, 25, 29). The pGLHB-*myb*, containing the human B-*myb* promoter driving the luciferase gene, has been described (14).

Cell Cycle Analysis—DNA content in the B-*myb* rescue experiments was analyzed by fluorescence-activated cell sorting. Briefly, 2 μg of the plasmid pCMV-CD20 were cotransfected with 0.5, 5, or 10 μg of pCMV-p107 or pCMV-pRb constructs and 10 μg of the wild type or mutant B-*myb* constructs in SAOS-2 cells. 48 h after transfections, cells were collected and stained with fluorescein isothiocyanate-labeled anti-CD20 antibody followed by propidium iodide staining, as described (25). For cell cycle studies performed in T98G cells, 5 μg of pCMV-CD20 plasmid were cotransfected with 20 μg of pCMV-B-*myb* or pCMV-empty plasmid. After 48 h, cells were collected and analyzed as described above. Cell cycle analysis of the GM cell lines was performed after 4 days of starvation in 0.5% fetal calf serum and subsequent restimulation with 10% fetal calf serum. At the end of the time-course the cells were fixed with 70% ethanol, labeled with propidium iodide, and analyzed by fluorescence-activated cell sorting using the software program Multicycle (Phoenyx Flow Systems).

Transient Transfections and Luciferase Assay—Transient transfections were carried out according to the calcium phosphate precipitation method (30). Briefly, 5 μg of B-*myb* promoter driving the luciferase cDNA were cotransfected with 1 μg of CMV-based constructs containing E2F1, E2F4, DP-1, pRb, and p107 in 10-mm dishes containing 2–5 $\times$ 10^5 cells each. Transfection efficiency was checked by including 1 μg of pSV-βGal plasmid in each experimental point. Luciferase assays were performed using 5–10 μl of total cell lysates (600 μl/dish),depending on β-galactosidase activity. Lysates were mixed with the luciferase substrate following the manufacturer's instructions (Promega). Luciferase activity was measured with the aid of a scintillation counter.

Western Blot—36 h after transfection 5 $\times$ 10^5 cells were lysed in SDS sample buffer, boiled, and loaded onto a 4–15% gradient acrylamide minigel (Bio-Rad). After electrophoresis, the proteins were electroblotted onto nitrocellulose, and the filter was then incubated with a 1:1000 dilution of monoclonal antibody XZ-37 (a kind gift of Dr. G. Condorelli), which recognizes the same epitope in both p107 and pRb. After several washes in phosphate-buffered saline, 0.1% Nonidet P-40 the filter was incubated with peroxidase-labeled secondary antibody and washed, and the signal was detected using the ECL reagent (Amersham Corp.).

RESULTS

B-myb Rescue of p107-induced G_1 Block—A large segment of the human and murine B-*myb* promoters share a high degree of homology and a site that has been shown to bind E2F, suggesting that the mechanism of B-*myb* transcriptional control involving E2F and its partners is evolutionally conserved. B-*myb* promoter activity peaks during the S phase of the cell cycle, and in both the murine and human systems, p107 can be detected in a cell cycle-regulated manner bound to the E2F site (14, 15, 28). To investigate the possibility that a functional link exists between p107 expression and B-*myb* activity, we co-transfected increasing amounts of CMV-driven p107 and pRB in SAOS-2 cells, which have been shown to be sensitive to the suppressive action of both proteins, and an expression vector driving B-*myb*. A CMV-CD20 plasmid was included in each transfection, and cells positive for CD20 were subjected to fluorescence-activated cell-sorting analysis. As expected, increasing amounts of both p107 and pRb resulted in a reduction in the number of cells traversing the S phase. However, when 10 μg of B-*myb* expression vector were added to the transfection mixtures containing p107, a significant increase in the percentage of cells in S phase was observed (Fig. 1A), whereas B-*myb* lacked any activity in pRb-blocked cells (Fig. 1*B*). A B-*myb* mutant lacking the DNA-binding domain was without effect (Fig. 1*A*), suggesting that the DNA-binding function is required in the rescue activity. Surprisingly, overexpression of B-*myb* did not affect cell cycle distribution of SAOS2 cells (Fig. 1*A*, and data not shown), although it could alter the growth characteristics of T98G cells (see below), indicating that B-*myb* promotion of cell growth is cell type-specific and that the rescue of p107 is not due to a nonspecific growth-related effect.

The possibility that B-*myb* might neutralize p107 by protein-protein interaction was explored *in vivo* by co-transfecting B-*myb* and p107 expression vectors in SAOS2 cells, followed by immunoprecipitation with p107 antibody and Western blot with a B-*myb*-specific antibody. No association was found between B-*myb* and p107, although we cannot exclude the possibility that a weak or unstable interaction went undetected due to the limits of this technique (data not shown).

p107 Efficiently Represses E2F-mediated Transactivation of the B-myb Promoter—The rescue experiments demonstrated that there is a functional interaction between B-*myb* and p107. We speculated that B-*myb* can rescue a p107-induced G_1 block because p107 function might be related to the inactivation of B-*myb* gene transcription, that is B-*myb* may be a downstream target of p107. In order to address this hypothesis directly, we co-transfected a luciferase vector driven by the human B-*myb* promoter and CMV-driven E2F1, E2F4, DP-1, and various retinoblastoma family members in different combinations. E2F1 induced a dramatic increase in luciferase activity when co-transfected with the B-*myb* promoter (Fig. 2*A*). E2F1-induced transactivation of the B-*myb* promoter in SAOS-2 cells was several times greater than that observed previously in the HaCaT cell line (14), perhaps reflecting the lack of functional pRb and very low levels of p107 and p130 in SAOS2 cells (Fig. 3, and data not shown). p107 caused a significant decrease (>10 fold) of E2F1-induced transactivation of the B-*myb* promoter, whereas pRb caused a 3–4-fold reduction (Fig. 2*A*). This effect was reproduced in three independent experiments, and a Western blot performed after transient transfection revealed that p107 and pRb proteins were both expressed at high levels, although pRb appeared to be more abundant than p107 (Fig. 3). E2F4-induced transactivation of the B-*myb* promoter was very weak as compared with that induced by E2F-1 (3–4-fold *versus* >300-fold), and required the presence of DP-1 (Fig. 2*B*). Significantly, the B-*myb* promoter activity was repressed substantially by co-transfection with either p107 or pRb alone (Fig. 2*C*). Thus, it is suggested that these proteins can affect transcription of B-*myb* by endogenous factors, consistent with the notion that B-*myb* is a downstream target of these negative regulators.

B-myb Induces G_1/S Transition in Human Glioblastoma Cells—The fact that B-*myb* can partially overcome p107-induced growth arrest suggests that B-*myb* can interfere with the cell cycle machinery of human cells. To investigate in detail the influence of B-*myb* on cell cycle progression we took advantage of the human glioblastoma cell line T98G, whose proliferation is strictly regulated by serum. After 4 days in medium containing 0.5% serum, approximately 90% of T98G cells were found blocked in G_0; feeding the cells with 10% serum causes the cells to undergo a synchronous round of replication. The cell cycle profiles of T98G cells constitutively expressing a transfected B-*myb* gene were found to differ markedly from parental cells upon reentry into cycle. Thus, the number of cells traversing the G_1/S border was significantly higher in T98G cells constitutively expressing B-*myb*. In fact, after 24 h of serum stimulation, 8% of cells overexpresssing B-*myb* were in G_1, and most of the cells appeared to reside in S phase (88.5%), with only 3.4% cells having reached G_2. In contrast, the parental cell line

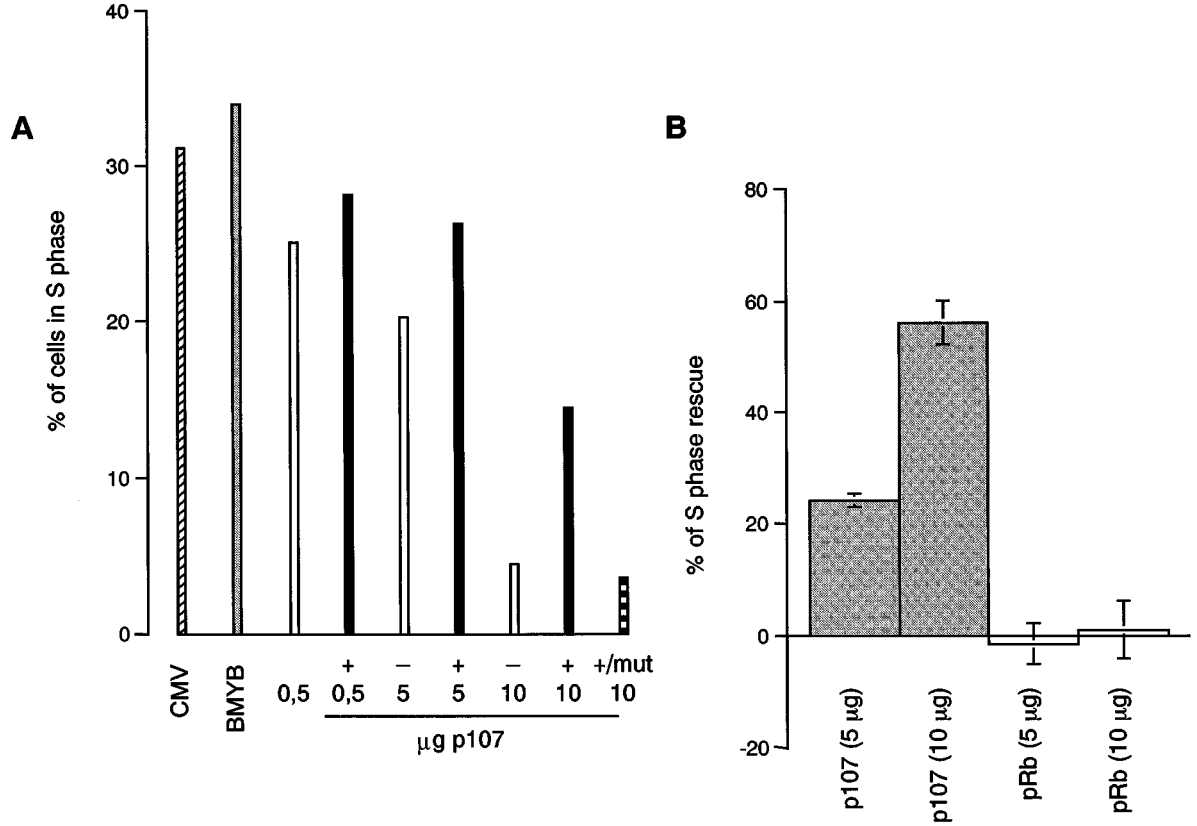

FIG. 1. **B-*myb* can partially rescue 107-induced block of DNA synthesis.** *A*, exponentially growing SAOS2 cells were transfected with control vector, CMV-Bmyb, or with a combination of increasing amounts of CMV-p107 plus (+) or minus (−) a fixed amount of CMV-Bmyb (10 μg). 10 μg of a CMV vector producing a B-*myb* mutant, lacking the DNA-binding domain (8), was also tested and is indicated as +/*mut*. A representative experiment is shown. *B*, quantitation of the percentage of cells reentering S phase in p107 + B-*myb* transfected cells as compared with pRb+ B-*myb* transfected cells. Cells transfected with p107 or pRb plus a CMV-empty vector provided the base-line values of S phase rescue. *Columns* represent the mean of three experiments, and the S.D. is indicated by *error bars*.

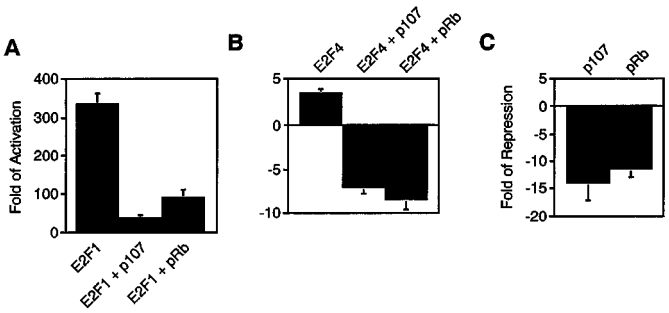

FIG. 2. **Basal activity and transactivation of the human B-*myb* promoter by E2F is inhibited by co-transfection with retinoblastoma family members in SAOS2 cells.** Extracts were made from cells 36 h following transfection, and luciferase assays were performed as indicated under "Materials and Methods." Data are expressed as -fold activation over the control. The mean of three independent experiments ± S.D. is shown. *A*, pGLHB-*myb* (5 μg) was cotransfected with 1 μg of CMV-E2F1 or CMV empty vector (promoter plus empty vector was used to determine the basal promoter activity) with or without CMVp107 or CMVpRb (5 μg each). *B*, pGLHB-*myb* (5 μg) was cotransfected with 1 μg of CMV-E2F4 + 1 μg of CMV-DP1 or CMV empty vector with or without CMVp107 or CMVpRb (5 μg each). *C*, pGLHB-*myb* (5 μg) was cotransfected with CMVp107 or CMVpRb (5 μg) or CMV empty vector.

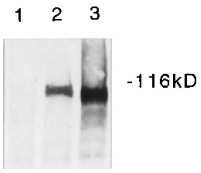

FIG. 3. **Expression levels of p107 and pRb proteins in SAOS2 cells following transient transfection with CMV-driven p107, pRb, and control vector.** The cells were collected 36 h after transfections, and equal amounts of cells were directly lysed in SDS sample buffer and subjected to polyacrylamide gel electrophoresis. After blotting the filter was stained with Poinceau Red to check for equal loading of the lanes and incubated with XZ-37 antibody. *Lane 1*, CMV; *lane 2*, p107; *lane 3*, pRb.

showed a percentage of cells in in G_1 of 35%, with 52.2 and 12.8% of cells in S and G_2, respectively (Fig. 4, *A* and *B*). This experiment was repeated with additional clones with identical results.

We also co-transfected T98G cells with pCMV-B-*myb* and the pCMV CD20 plasmids followed by cell cycle analysis after 48 h. Enforced expression of B-*myb* in asynchronously growing cells reduced the percentage of cells in G_1, while the percentage of cells in S phase was increased (Table I). Although the extent of S phase induction in this experiment does not appear to be dramatic, we should consider that at the time of transfection these cells are actively proliferating and that the degree of stimulation is comparable with that obtained by ectopic expression of E2F4 in SAOS2 cells under similar experimental conditions (19).

DISCUSSION

Recent evidence indicates that the different products of the retinoblastoma family participate in cell cycle control, performing similar yet distinct functions as the cell progresses from G_0 to mitosis. A model has been developed that shows, depending on the phases of the cell cycle, different retinoblastoma family members in association with E2F. The complexity is even greater if we take into account that five different E2F genes

A

GM-47

0 h- 87%(G1)	7.8% (S)	5.1% (G2)
12 h-92.8	4.4	2.8
16 h-50.7	47.9	1.4
20 h-37.1	59.6	3.3
24 h-35.0	52.2	12.8
48 h-83.0	9.4	7.7

GM-B-Myb

0 h- 90.1%(G1)	6.0% (S)	4.0% (G2)
12 h-92.8	4.0	3.1
16 h-55.6	41.0	3.3
20 h-18.8	80.8	0.5
24 h-8.2	88.4	3.4
48 h-81.1	9.0	9.9

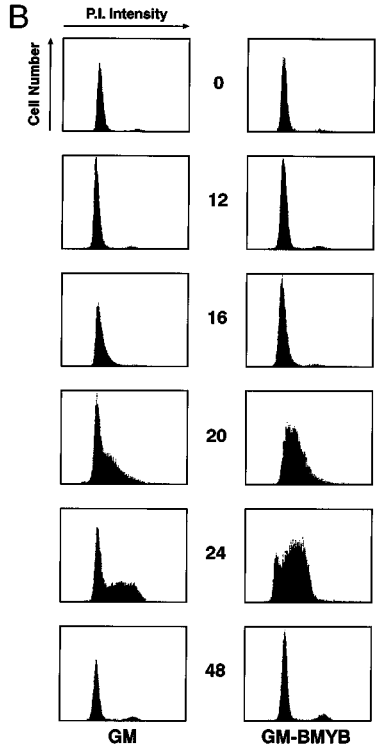

FIG. 4. **Cell cycle profiles of the human glioblastoma GM-47 cell line (A T98G subline) and the derivative GM-B-myb cell line at various time points after serum stimulation.** *A*, quantitative analysis; *B*, graphic display of the data shown in *A*.

have been cloned, whose encoded products can interact with two and maybe more DP proteins (27). It has been shown that several growth-regulated genes contain an E2F site located in close proximity to the transcription start site (31). Some, but not all, of these promoters are activated by E2F1 (14, 30) and it is thought that interaction of E2F with retinoblastoma family members can repress E2F-induced transactivation, switching the E2F site from a positive to a negative element (28, 32).

The rationale of the present study is based on experiments that demonstrated the presence of cell cycle-regulated complexes containing distinct retinoblastoma family members bound to the E2F site of the human and mouse B-*myb* promoters. In particular, it was shown that p107 containing complexes present during G_1 and S phase on the murine B-*myb* promoter are disrupted by the E7 oncoprotein, resulting in the activation of B-*myb* transcription (15). These experiments suggested that p107 might be specifically required to modulate growth-regulated activity of the B-*myb* promoter and that p107-induced growth arrest may be due, at least in part, to repression of B-*myb* transcription. B-*myb* is required for the proliferation of several human and murine cell lines, it is broadly expressed, and its overexpression induces altered growth characteristics of murine Balb/c 3T3 cells (7). We have therefore investigated the possibility that B-*myb* and p107 may be functionally linked. B-*myb* overexpression induces S phase in cells that have been blocked by p107 (Fig. 1*A*); this effect seems to be specific because B-*myb* cannot rescue the block induced by pRb (Fig. 1*B*). To test directly the hypothesis that p107 may be involved in the control of B-*myb* transcription, we analyzed the activity of the human B-*myb* promoter in the presence of E2F in combination with p107 or pRb. The human B-*myb* promoter is activated during the G_1/S transition of the cell cycle, whereas it is silent during G_0 and early G_1 (14). We found that p107 was more effective than pRb in the inhibition of E2F1-mediated activation of the B-*myb* promoter (Fig. 2*A*). This effect is even more impressive if we take into account that pRb is expressed at higher level than p107 in transiently transfected SAOS2 cells (Fig. 3). On the other hand, both p107 and pRb appeared to possess similar activity in the inhibition of E2F4-induced transactivation, which was very weak with respect to that of E2F1 (Fig. 2*B*). Based on these experiments and previous data, we can hypothesize that E2F1 and/or other G_1/S-induced E2Fs are involved in the activation of the B-*myb* gene during S phase, whereas E2F4, which is thought to be associated with the G_0/G_1 transition of the cell cycle (33), might rather be involved in the repression of B-*myb* expression during G_0 and early G_1.

The evidence provided in this study strongly suggests that p107 might be required to modulate E2F-mediated activation of the B-*myb* promoter. There is no indication to date of a direct interaction between p107 and E2F1 *in vivo*, although these proteins do associate when overexpressed (19). Our finding that p107 was more efficient than pRb in the inhibition of E2F1-mediated transactivation of a physiological promoter (B-*myb*) might be the consequence of enforced expression of the retinoblastoma family members; alternatively, it would suggest that the weak interaction between p107 and E2F1 is functionally relevant. An important finding in this study was that B-*myb* promotes S phase in a human cell line, thus defining a cause/effect relationship between B-*myb* expression and DNA synthesis. In fact, B-*myb* overexpression induced lengthening of the S phase and thus massive accumulation of glio-

TABLE I

Cell cycle kinetics of T98G cells transiently transfected with pCMV, pPCMV-B-myb, and PCMV-CD20 plasmids

Transfection and cell cycle analysis were performed as described under "Materials and Methods." This experiment was performed in duplicate with comparable results.

Sample	G_1	S	G_2
	%	%	%
T98G	46	38	16
T98G-PCMV 1	41	34	25
T98G-PCMV 2	42	35	23
T98G-BMYB 1	35	42	23
T98G-BMYB 2	36	46	18
T98G-BMYB 3	36	43	21
T98G-BMYB 4	35	42	23

blastoma cells in this phase after the addition of serum (Fig. 4); B-*myb* was not able to override the G_0 arrest imposed by serum deprivation, indicating that its function can be exerted exclusively during S phase. At least some B-*myb* functions appear to be cell type-dependent; overexpression of B-*myb* did not induce enhanced proliferation of SAOS2 cells, nor of other murine cell lines (34). Indeed, in a recent report it was shown that B-*myb* transcriptional activity is cell type-dependent and requires a specific co-factor (6).

Three different classes of growth suppressor agents (p107, p53, and TGF-β) seem to target expression levels of the B-*myb* gene (Fig. 2 and Refs. 8, 13, and 16). Conversely, overexpression of B-*myb* appears to overcome the block induced by both p53 and p107 (Ref. 8 and Fig. 1). This is suggestive of a possible involvement of B-*myb* in human tumorigenesis. The mechanism(s) underlying the positive action of B-*myb* on cell cycle progression probably rests in the activation of genes required for DNA synthesis. We previously showed that B-*myb* induces up-regulation of genes involved in cell cycle control such as cyclin D1 and *cdc2* in murine Balb/c 3T3 cells (7). Cyclin D1 is required for cell proliferation and, when overexpressed, shortens the G_1 phase of the cell cycle (35, 36), whereas *cdc2* is required for DNA synthesis and mitosis (37, 38). The identification of B-*myb* molecular targets would represent the next step needed in understanding its role in the regulation of the cell cycle.

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