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Regulation of BALB/c 3T3 fibroblast proliferation by B-myb is accompanied by selective activation of *cdc2* and cyclin D1 expression

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ABSTRACT **The B-*myb* gene is expressed in many cell types at the G_1/S transition of the cell cycle. Inhibition of B-*myb* expression in BALB/c 3T3 fibroblasts by introduction of a B-*myb* antisense construct greatly diminished cell proliferation, whereas constitutive expression of a human B-*myb* cDNA in these cells reduced their growth factor requirements and induced a transformed phenotype. Constitutive expression of B-*myb* cDNA was accompanied by activation of cyclin D1 and *cdc2* expression but not of cyclin A and cyclin B. Transfection of BALB/B-myb cells (a cell line expressing high levels of exogenous human B-*myb*) with a cyclin D1 antisense construct drastically reduced cloning efficiency of these cells. These results suggest that the B-*myb*-encoded product regulates fibroblast proliferation by activating *cdc2* and cyclin D1 gene expression and that abnormal expression of cyclin D1 might be a step in the process of transformation.**

The A-*myb* and B-*myb* genes, which are related to the protooncogene c-*myb*, the cellular homolog of the transforming gene of the avian myeloblastosis virus, and the avian leukemia virus E26, which cause myeloblastic leukemia in chickens and transform myelomonocytic hematopoietic cells in culture (1, 2), have been isolated.

Recent evidence suggests that c-*myb* plays an important role in the regulation of normal and leukemic hematopoiesis and T-lymphocyte proliferation (3–7). Inhibition of c-*myb* expression prevents G_1/S transition in leukemic cell lines and normal T lymphocytes and is associated with selective down-regulation of DNA polymerase α expression (8), suggesting the direct involvement of c-myb in an essential biochemical pathway leading to DNA synthesis. Moreover, constitutive expression of human c-*myb* in murine fibroblasts abrogates the requirement for the cell cycle progression factor insulin-like growth factor 1 (9), suggesting a general role for c-myb in cellular proliferation.

B-myb is homologous to c-myb in the DNA binding domain, and its pattern of expression is not restricted to hematopoietic cells (10). In fact, B-myb transcripts have been detected in the majority of cell lines and tissues tested (10). Recent studies have shown that, like c-myb, B-myb acts as a transactivating factor; in transient expression assays, a constitutively expressed B-*myb* cDNA transactivates a reporter gene linked to the simian virus 40 (SV40) early promoter and enhancer through interaction of the encoded protein with B-myb binding sites in the SV40 early promoter (11).

B-myb is also expressed in BALB/c 3T3 fibroblasts at the G_1/S boundary with kinetics similar to that of c-myb in phytohemagglutinin-stimulated normal T lymphocytes (12, 13). Inhibition of B-*myb* expression by low concentrations of the protein synthesis inhibitor cycloheximide and at the restrictive temperature in G_1-specific temperature-sensitive mutants of the cell cycle (13) supports the notion that B-myb is growth-regulated at the G_1/S boundary. Because c-myb is not expressed at detectable levels in fibroblasts, we asked whether B-myb might be the functional equivalent of c-myb in regulating proliferation in nonhematopoietic cells. We used the model system of BALB/c 3T3 fibroblasts to study cell proliferation because they have a well-defined growth factor requirement (14, 15), which allows determination of the stage in the G_0/S transition that requires B-myb function. We report here that B-myb is needed for BALB/c 3T3 proliferation, perhaps by selectively activating the expression of *cdc2* and cyclin D1 genes.

MATERIALS AND METHODS

Cloning of a Full-Length B-*myb* cDNA. The full-length B-*myb* cDNA was cloned as follows. A human lymphoma cDNA library cloned in the λgt11 vector was screened with a 1.4-kilobase radiolabeled B-*myb* fragment (10). The longest recombinant phage was used to release a 1469-base-pair (bp) fragment spanning the *Eco*RI site 3′ of the polyadenylylation signal to an *Eco*RI site at nucleotide 1161 of the published B-*myb* cDNA sequence. This fragment was subcloned into an SK plasmid vector (Stratagene). The remaining 5′ portion of the cDNA was cloned by PCR amplification of reverse-transcribed B-*myb* mRNA from HL-60 cells. The cloning reaction was carried out in three different steps:

(*i*) B-*myb* cDNA extending from nucleotide 749 to nucleotide 1235 was amplified and subcloned into an *Sma* I-digested SK vector and ligated to the previously subcloned B-*myb* by utilizing a *Ban* I site (nucleotide 1219).

(*ii*) A longer B-*myb* segment extending from nucleotide 197 to nucleotide 1127 was amplified and subcloned into an *Sma* I-digested SK vector. Ligation of the two B-*myb* portions at an *Xho* I site (nucleotide 743) resulted in a B-*myb* clone that lacked the first 69 bp starting from the ATG initiation site.

(*iii*) Two oligomers covering the remaining portion of the cDNA were synthesized since amplification of this portion by PCR was unsuccessful. The B-*myb* cDNA segments obtained by PCR or by synthetic oligomers were sequenced and found to be identical to the published sequence. The full-length B-*myb* cDNA was subsequently eluted from the SK vector (pSKB-myb1), digested with *Cla* I and *Xba* I, and subcloned into the pSV40 polylinker vector, which contains a polycloning site located between the pSV40 early promoter and the SV40 polyadenylylation signals, and designated pSV/B-myb.

Cloning of an Antisense B-*myb* Construct. The construct pSV/anti-B-myb contains a 2-kilobase B-*myb* cDNA fragment cloned in the antisense orientation with respect to the SV40 promoter. To obtain the construct, pSKB-myb1 was linearized by *Xho* I digestion; then the restriction site was filled in by treatment with the Klenow fragment of DNA polymerase I before digesting with *Spe* I. The B-*myb* frag-

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Abbreviation: SV40, simian virus 40.

ment (*Xho* I-blunted–*Spe* I) was cloned into the pSV40 polylinker vector linearized with *Xba* I, filled in with the Klenow fragment, and subsequently digested with *Spe* I.

Cloning of an Antisense Cyclin D1 Construct. The construct pSV/anti-cyclin D1 contains a 606-bp cyclin D1 cDNA fragment cloned in the antisense orientation with respect to the SV40 promoter and was obtained as follows: a 606-bp cyclin D1 cDNA fragment (from nucleotide 188 to nucleotide 743) was synthesized by reverse transcription–PCR from BALB/c 3T3 RNA with a 5′ primer (5′-ATGGAACAC-CAGCTCCTG-3′) and a 3′ primer (5′-CATGGAGGGTGGC-TGGAAAT-3′) derived from the published murine cDNA sequence (16) and cloned into the vector PCR 1000 (Invitrogen). Sequence analysis revealed that the cloned fragment was identical to the published murine cyclin D1 sequence (16).

The cyclin D1 fragment was subsequently eluted from the vector PCR 1000 by digestion with *Spe* I and *Eco*RI restriction enzymes and cloned in the antisense orientation into the pSV40 polylinker vector digested with *Eco*RI and *Xba* I.

Cloning of a Dexamethasone-Inducible Antisense Cyclin D1 Construct. The *Spe* I–*Eco*RI 0.6-kilobase cyclin D1 fragment was made blunt-ended with the Klenow fragment and ligated in the antisense orientation with respect to the dexamethasone-inducible mouse mammary tumor virus promoter of the plasmid vector pMAMneo (Clontech), linearized with *Sal* I, and filled in with the Klenow fragment.

Cell Lines. The original cell line from which all the other cell lines have been derived is BALB/c 3T3, a kind gift of Renato Baserga (Dept. of Microbiology, Thomas Jefferson University, Philadelphia). Derivatives of BALB/c 3T3 cell lines were generated by transfection.

Growth of Cells in Mass Culture. BALB/c 3T3 or derivative cell lines were plated at a concentration of 10^4 cells per plate in 60-mm Petri dishes with 10 ml of 10% (vol/vol) bovine serum (5% calf serum plus 5% fetal calf serum) or 1% calf serum. The growth medium was Dulbecco's modified Eagle's medium (DMEM).

Soft Agar Assay. The basal layer (8 ml) was made with 10% bovine serum and DMEM containing 1.5% Noble agar and was incubated at 37°C for 12 hr. The top layer (1.5 ml) contained 10% serum, DMEM, and 0.3% Noble agar. The cells were seeded at 1000 per 60-mm Petri dish. Foci were counted 10–14 days after the cells were seeded.

Transfection. Plasmids (10 μg per 10^6 cells) were transfected into BALB/c 3T3 cells by calcium phosphate precipitation in the presence of 1 μg of pLHL4, which carries the hygromycin-resistance gene (17).

RNA Blots. RNA was extracted by the method of Chomczynski and Sacchi (18) and analyzed on RNA blots by the method of Thomas (19).

Reverse Transcription–PCR Phenotyping of BALB/B-myb Cells. Levels of cyclin A, cyclin B, cyclin D1, *cdc2*, and β-actin mRNAs were measured by reverse transcription–PCR as described (8), except that cyclin A and cyclin B amplifications were achieved at the annealing temperature of 37°C. The cyclin A 5′ primer (nucleotides 753–770 of human cyclin A cDNA) was 5′-GACTGGTTAGTTGAAGTA-3′; the 3′ primer (nucleotides 975–992) was 5′-CTCCATTCT-CAGAACTTG-3′; the oligonucleotide probe extended from nucleotide 861 to nucleotide 884; all are from the published sequence (20). The cyclin B 5′ primer (nucleotides 780–797 of the human cyclin B cDNA) was 5′-TAGTTCCATCAGG-TATTTGGC-3′; the 3′ primer (nucleotides 1201–1221) was 5′-TAGTTCCATCAGGTATTTGGC-3′; the oligonucleotide probe extended from nucleotide 1141 to nucleotide 1164; all are from the published sequence (21). The cyclin D1 5′ primer (nucleotides 590–613 of mouse cyclin D1 cDNA) was 5′-GAGGAGCTGCTGCAAATGGAACTG-3′; the 3′ primer (nucleotides 773–793) was 5′-CATGGAGGGTGGGTTG-GAAAT-3′; the diagnostic oligomer probe extended from nucleotide 659 to nucleotide 688; all are from the published sequence (16). The *cdc2* 5′ primer (nucleotides 54–75) was 5′-GGAGAAGGTACCTATGGAGTTG-3′; the 3′ primer (nucleotides 307–328) was 5′-GAATCCATGTACTGAC-CAGGAG-3′; the synthetic probe was from nucleotide 147 to nucleotide 167; all are from the published sequence (22). The β-actin 5′ primer (nucleotides 224–244) was 5′-TGG-GAATGGGTCAGAAGGACT-3′; the 3′ primer (nucleotides 412–433) was 5′-TTTCACGGTTGGCCTTAGGGTT-3′; the synthetic probe was from nucleotide 258 to nucleotide 236; all are derived from the published sequence of mouse β-actin cDNA (23).

***In Vitro* Transcription and Translation of B-*myb* cDNA.** The plasmid template (pSKB-myb1) was linearized either by *Not* I or by *Eco*RV restriction enzymes to obtain the sense or the antisense B-*myb* mRNA, respectively. Capped mRNA was synthesized by using T7 or T3 polymerase, respectively, as suggested by the manufacturer (Stratagene). *In vitro* translation was performed by incubating 1 μg of capped mRNA with rabbit reticulocyte lysate and ^{35}S according to the instructions of the manufacturer (Promega).

[^{3}H]Thymidine Incorporation Assay in Dexamethasone-Treated BALB/B-myb Cells. Cells (1×10^5 per well) were

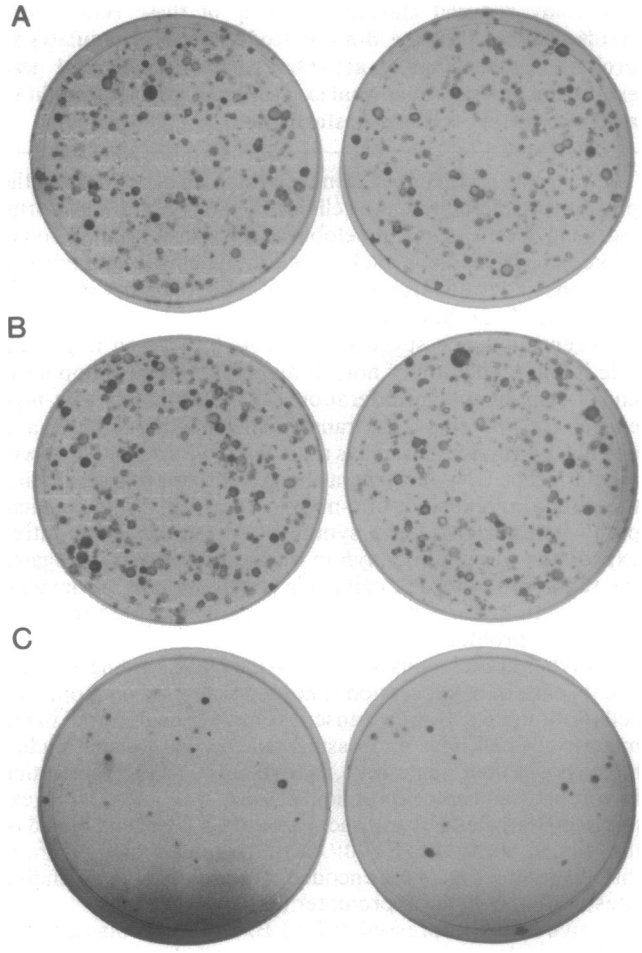

FIG. 1. Cloning efficiency of BALB/c 3T3 cells transfected with a pSV/anti-B-myb construct. Plasmids (10 μg per 10^6 cells) were transfected into BALB/c 3T3 cells by calcium phosphate precipitation in the presence of 1 μg of pLHL4, which carries the hygromycin-resistance gene (17). Hygromycin-resistant clones were selected in hygromycin-containing medium (250 μg/ml), scored 12 days after transfection, and stained with crystal violet. (*A*) pSV40 polylinker-transfected cells. (*B*) pSV/B-myb1-transfected cells. (*C*) pSV/anti-B-myb-transfected cells.

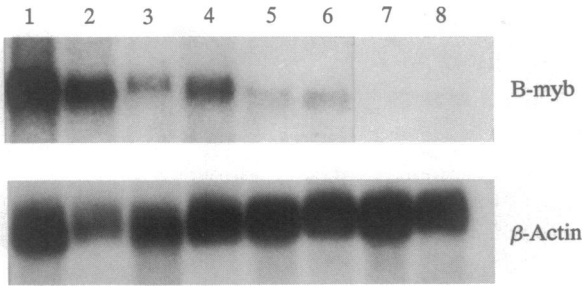

FIG. 2. Levels of expression of endogenous murine B-*myb* mRNA in BALB/c cells transfected with a B-*myb* antisense construct. Total cellular RNA was extracted from individual cell clones obtained as described in Fig. 1 and subjected to Northern blot analysis. Lanes 1–4, BALB/c 3T3 clones obtained after transfection with the polylinker vector; lanes 5–8, BALB/c 3T3 clones obtained after transfection with the B-*myb* antisense construct.

plated in 24-well plates, and the next day the cells were treated with 1 μM dexamethasone. Four hours later [^{3}H]thymidine was added at 1 μCi (1 Ci = 37 GBq) per well, and the cells were collected 20 hr later on Millipore glass filters and washed three times with cold 10% (vol/vol) trichloroacetic acid. Filters were dissolved in vials containing filter scintillation fluid and cpm were assayed on a Beckman scintillation counter.

RESULTS

Cloning Efficiency of BALB/c 3T3 Cells Transfected with an Antisense B-*myb* Construct. Analysis of cloning efficiency of BALB/c 3T3 cells cotransfected with an antisense B-*myb* construct driven by the SV40 early promoter and a hygromycin-resistance gene (10:1 ratio) followed by selection in hygromycin-containing medium revealed a drastically reduced number of hygromycin-resistant colonies (≈90% inhibition) as compared to BALB/c 3T3 cells transfected with the vector only (Fig. 1). This experiment was repeated three times with different plasmid preparations; furthermore, levels of the endogenous B-*myb* transcripts were barely detectable in residual B-myb antisense transfectants (Fig. 2). Together, the data suggest a specific effect of the B-*myb* antisense construct on cell proliferation and indicate that B-*myb* is required in normal fibroblast proliferation.

Growth Characteristics of BALB/c 3T3 Cells Constitutively Expressing B-*myb*. To examine the effects of B-*myb* constitutive expression on the growth of BALB/c 3T3 cells, a cell line (BALB/B-myb) expressing high levels of exogenous human B-*myb* was established by transfecting those cells with the plasmid pSV/B-myb1. Under high-stringency hybridization conditions, a positive signal corresponding to the human B-*myb* mRNA was detected with the human probe in lanes containing RNAs derived from BALB/B-myb and HL-60 cells, but not from the parental BALB/c 3T3 cells (Fig. 3*A*). Antibodies against the B-myb protein are still not available, so detection of the B-myb protein in transfected cells is precluded. However, to determine whether our construct would at least be translated *in vitro*, RNA transcribed from the construct pSKB-myb1 was incubated *in vitro* with a reticulocyte lysate; a protein of about 80 kDa was generated

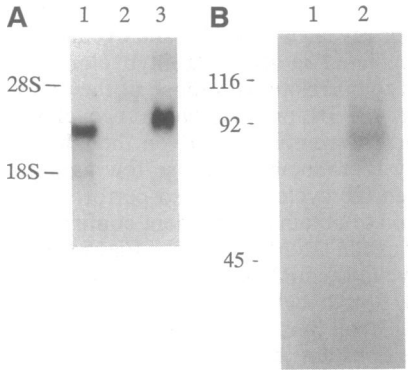

FIG. 3. (*A*) Expression of human B-*myb* mRNA in BALB/c 3T3 fibroblasts. BALB/c 3T3 fibroblasts were maintained in culture by serial passages in DMEM supplemented with 10% bovine serum and transfected by the calcium phosphate procedure with pSV/B-myb1 or with the plasmid pSV polylinker vector along with the hygromycin-resistance plasmid pLHL4 (17). After 12–16 days of selection in hygromycin-containing DMEM (250 μg/ml), mixed cell populations or single clones were picked up and maintained in culture under the same conditions of selection. RNAs from transfected cell lines and HL-60 cells were obtained as described (18). Total cellular RNA (10 μg) was electrophoresed in an agarose/formaldehyde gel, transferred to nylon filters, and hybridized to a radiolabeled human B-*myb* cDNA fragment as described (19). Lane 1, HL-60 cells; lane 2, BALB/c 3T3 cells carrying the pSV40 polylinker vector; lane 3, BALB/c 3T3 cells carrying the construct pSV/B-myb1. (*B*) *In vitro* transcription and translation of B-*myb* cDNA. After *in vitro* transcription and translation of B-*myb* cDNA, one-fifth of the reaction mixture was run on an SDS/10% polyacrylamide gel, dried, and autoradiographed. Lane 1, translation product of B-*myb* antisense RNA (negative control); lane 2, translation product of B-*myb* sense mRNA.

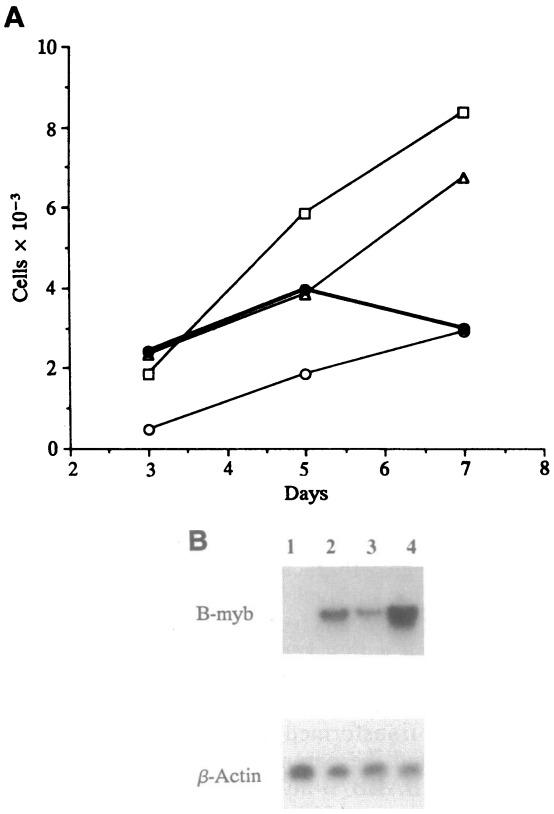

FIG. 4. (*A*) Growth of BALB/B-myb clones in 1% fetal calf serum. Cells (2 × 10^3 per well) were plated in DMEM containing 1% fetal calf serum in 24-well Costar plates; cell number was evaluated by counting the cells from duplicate wells with the aid of a hemocytometer after trypsinization. △, BALB/B-myb clone 1; ○, BALB/B-myb clone 2; □, BALB/B-myb clone 3; ●, control. (*B*) Expression of B-*myb* mRNA in BALB/B-myb clones. Total RNA was processed for hybridization to a human B-*myb* cDNA fragment as described in the legend to Fig. 1. Lane 1, BALB/c 3T3 cells transfected with the SV40 polylinker vector; lane 2, BALB/B-myb clone 1; lane 3, BALB/B-myb clone 2; lane 4, BALB/B-myb clone 3. β-Actin served as control.

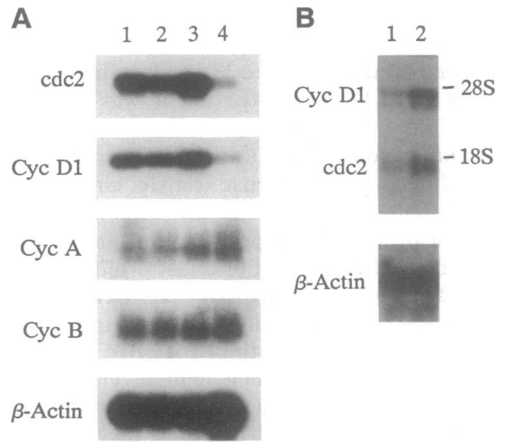

FIG. 5. Expression of *cdc2* and cyclin genes in BALB/B-myb cells. (*A*) BALB/B-myb clones were plated in DMEM containing 10% bovine serum, and RNAs were extracted during exponential growth. Lane 1, BALB/B-myb clone 1; lane 2, BALB/B-myb clone 2; lane 3, BALB/B-myb clone 3; lane 4, BALB/c 3T3 transfected with the pSV40 polylinker vector. (*B*) Culture conditions and RNA extraction of parental BALB/c 3T3 and BALB/B-myb cell lines were as described above. Ten micrograms of total cellular RNA from each sample was electrophoresed in an agarose/formaldehyde gel and analyzed for *cdc2* and cyclin D1 mRNA expression by the Northern blot technique. Lane 1, BALB/c 3T3 cells; lane 2, BALB/B-myb cells.

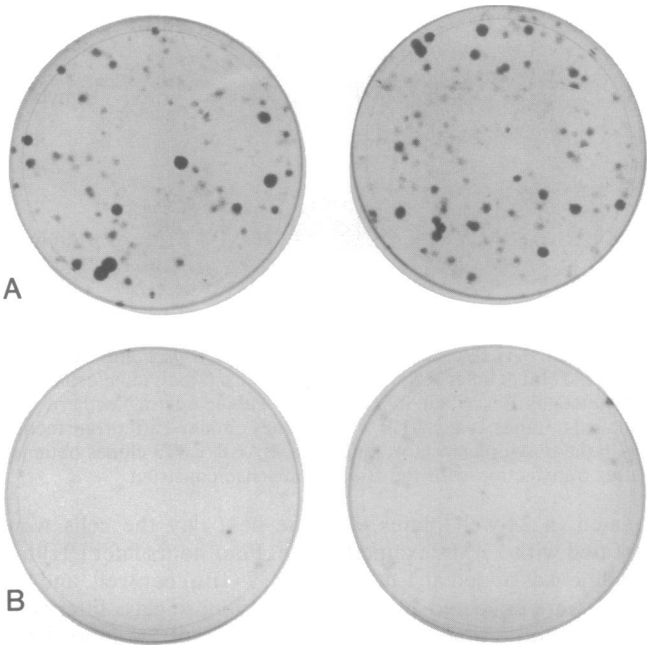

FIG. 6. Cloning efficiency of BALB/B-myb cells transfected with a pSV/anti-cyclin D1 construct. Plasmids were transfected into BALB/B-myb cells (10 μg per 10^6 cells) by calcium phosphate precipitation in the presence of 1 μg of pRSV-Neo, which carries the neomycin-resistance gene. Colonies were scored after 14 days of selection in medium containing G418 (800 μg/ml) and stained by crystal violet. (*A*) pSV40 polylinker-transfected cells. (*B*) pSV/anti-cyclin D1-transfected cells.

(Fig. 3*B*), which is in good agreement with the size predicted from the cDNA sequence. To determine whether B-*myb* expression could alter the growth characteristics of the transfected cells, we plated BALB/B-myb and control BALB/c 3T3 cells transfected with the pSV40 vector lacking the B-*myb* cDNA in medium containing 1% serum, which induces entry into a quiescent state. After 9 days of culture, the number of BALB/B-myb cells was significantly higher than that of the control cells (≈12×10^4 cells per well in 24-well plates for BALB/B-myb as compared to ≈3×10^4 cells of BALB/c 3T3 transfected with the vector alone), indicating that BALB/B-myb cells can still proliferate in low serum conditions, although at a reduced rate (doubling time of 48 hr in 1% serum as compared to 24 hr in 10% serum). The reduced serum requirements of randomly picked single clones from different transfections correlated well with mRNA levels of exogenous B-*myb*, as indicated by the capacity of clones with high B-*myb* expression to grow in 1% serum (Fig. 4 *A* and *B*), whereas BALB/c 3T3 cells require platelet-derived growth factor and insulin or insulin-like growth factor 1 for survival in serum-free medium. BALB/B-myb cells not only survived but also grew in serum-free medium, although with a very slow (≈96 hr) doubling time (data not shown). To determine whether the growth-factor independence of BALB/B-myb was an indication of progression toward a transformed phenotype, we plated both BALB/B-myb and the parental cells in soft agar. After 10 days of culture, BALB/B-myb cells formed colonies in agar with high efficiency (≈50%), whereas BALB/c 3T3 cells formed small colonies, with an efficiency of only 1–5% (data not shown).

Expression of *cdc2* and Cyclin Genes in BALB/c 3T3 Cells Constitutively Expressing B-*myb*. To explore the potential molecular mechanisms associated with the growth factor-independent and transformed phenotype of BALB/B-myb cells, we analyzed the effect of constitutive B-*myb* expression on *cdc2* and cyclin genes since they appear to be important both at the G_1/S transition and during mitosis (16, 24–28). In addition to cyclin A and cyclin B, we focused on the newly identified cyclin D1 gene, because it appears to have a role in G_1 in mammalian cells and might be important in oncogenesis in light of its identity with *PRAD1*, a gene rearranged in parathyroid tumors and genetically linked to the B-lymphocytic leukemia-associated *bcl-1* locus (25, 29). PCR analysis revealed that *cdc2* and cyclin D1 mRNA levels were up-regulated in BALB/B-myb clones (Fig. 5*A*) and were found to correlate with those of the exogenous human B-*myb* (Fig. 4*B*). In contrast, cyclin A, cyclin B, and β-actin mRNA levels were constant. Even though the PCR results were highly reproducible because few amplification cycles were utilized (20 cycles), we also performed Northern blot hybridization to obtain independent confirmation of changes in *cdc2* and cyclin D1 mRNA levels. Fig. 5 *B* and *C* shows that *cdc2* and cyclin D1 mRNA levels were markedly increased in the BALB/B-myb cell line as compared to the parental BALB/c 3T3 cell line. In contrast, cyclin A and B mRNA levels were constant (not shown). Thus, the effects of constitutive B-*myb* expression on *cdc2* and cyclin D1 expression appear to be specific.

Cloning Efficiency of BALB/B-myb Cells Transfected with an Antisense Cyclin D1 Construct. To determine whether cyclin D1 expression was required for the maintenance of the phenotype induced by B-*myb*, BALB/B-myb cells were transfected with an antisense cyclin D1 construct (10:1 ratio) driven by the SV40 early promoter in the presence of the gene encoding the resistance to neomycin. After selection in G418-containing medium, the number of G418-resistant colonies formed after transfection with the cyclin D1 antisense construct was drastically reduced (by ≈80%) as compared to BALB/B-myb cells transfected with the vector only (Fig. 6). Levels of cyclin D1 mRNA in cyclin D1 antisense-transfected cells were significantly reduced in comparison to those in cells transfected with vector only (data not shown). The need of cyclin D1 expression for cell cycle progression of BALB/B-myb cells was also demonstrated by transfecting in these cells a dexamethasone-inducible cyclin D1 antisense construct. After treatment with 1 μM dexamethasone, [^{3}H]thy-

Table 1. [^{3}H]Thymidine incorporation in the BALB/B-myb cell line transfected with a dexamethasone-inducible antisense cyclin D1 construct (pSV/anti-cyclin D1)

Cell line	Dex	cpm, mean ± SD	% inhibition	P (t test)
BALB/B-myb transfected with pSV/anti-cyclin D1	−	100,037 ± 21,756	0	
	+	33,679 ± 5,213	67	0.012
BALB/B-myb	−	75,674 ± 19,486	0	
	+	63,899 ± 14,006	16	0.44

Dex, dexamethasone.

midine incorporation was significantly inhibited in BALB/B-myb cells transfected with the inducible antisense cyclin D1 construct but not in nontransfected BALB/B-myb cells (Table 1).

DISCUSSION

In the present investigation, we assessed the role of B-myb in fibroblast proliferation by blocking the function of the endogenous B-*myb* mRNA in BALB/c 3T3 cells transfected with a human B-*myb* antisense construct and by studying the effects of B-*myb* constitutive expression on the growth of BALB/c 3T3 cells. The B-*myb* antisense construct inhibited the proliferation of BALB/c 3T3 cells, whereas constitutive B-*myb* expression was associated with reduced growth factor requirements and growth in soft agar. This phenotype was also induced by constitutive expression of the hybrid gene consisting of the DNA binding domain of B-*myb* and the transactivation domain of c-*myb* (data not shown), suggesting that B-myb exerts its function upon binding to the target genes. In this regard, the selective up-regulation of *cdc2* and cyclin D1 mRNA levels in BALB/c 3T3 cells constitutively expressing B-*myb* indicates that there is a functional link between B-myb and *cdc2* and cyclin D1 genes and raises the possibility of a direct interaction of B-myb protein with *cdc2* and/or cyclin D1 regulatory elements. Antisense oligodeoxynucleotides targeted against *cdc2* mRNA were previously shown to inhibit G_1/S transition in phytohemagglutinin-stimulated T lymphocytes (28). In addition, in that model of cell proliferation, *cdc2* mRNA expression is a late event and follows the appearance of c-*myb* mRNA (28); of even greater interest, treatment of phytohemagglutinin-stimulated T lymphocytes with c-*myb* antisense oligodeoxynucleotides prevents the late induction of *cdc2* mRNA expression (28). Because B-*myb* has several properties of a potential c-*myb* equivalent in fibroblast proliferation, the reported functional link between c-myb and cdc2 in T lymphocytes supports our own data of the link between B-myb and cdc2 in BALB/c 3T3 fibroblasts. The importance of the B-myb–cyclin D1 functional link was directly demonstrated by the ability of a cyclin D1 antisense construct to inhibit the cloning efficiency of BALB/B-myb cells (Fig. 6). It has been recently shown that cyclin A is required for the onset of DNA replication in mammalian fibroblasts (30), raising the possibility that the effects seen with the cyclin D1 antisense construct might have been caused by inhibition of cyclin A expression. Comparison of the DNA sequence of the cyclin D1 segment used in our studies with cyclin A cDNA sequence revealed only a 39% nucleotide homology (data not shown), which suggests that the antiproliferative effect seen with the cyclin D1 antisense construct was specific. These findings do not exclude the participation of other genes in the regulation of normal fibroblast proliferation by B-*myb* or in the abnormal growth associated with its constitutive expression. They provide, however, compelling evidence for the essential role of cyclin D1. Although the mechanism by which cyclin D1 regulates cell cycle progression is not known, it is likely that the cyclin D1-encoded protein interacts with and activates the $p34^{cdc2}$ kinase previously shown to regulate the G_1/S transition (28, 31). Alternatively, cyclin D1-encoded protein could also interact with related members of the cdc2 family, which could also be involved in the regulation of cell cycle progression (16, 32) to regulate the G_1/S transition. This would suggest the existence of multiple regulatory networks in cell cycle progression involving members of the *cdc2* and cyclin gene family. Finally, our findings support the possibility that cyclin D1 expression plays a direct role in the process of cellular proliferation and might be involved in neoplastic transformation.

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1. Moscovici, C. (1975) *Curr. Top. Microbiol. Immunol.* **71**, 79–96.
2. Moscovici, C. & Gazzolo, L. (1982) *Advances in Viral Oncology, Vol. 1 Oncogene Studies* (Raven Press, New York), pp. 83–106.
3. Gewirtz, A. M. & Calabretta, B. (1988) *Science* **242**, 1303–1306.
4. Caracciolo, D., Venturelli, D., Valtieri, M., Peschle, C., Gewirtz, A. M. & Calabretta, B. (1990) *J. Clin. Invest.* **85**, 55–61.
5. Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J. & Potter, S. S. (1991) *Cell* **65**, 677–687.
6. Anfossi, G., Gewirtz, A. M. & Calabretta, B. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3379–3383.
7. Gewirtz, A. M., Anfossi, G., Venturelli, D., Valpreda, S., Sims, R. & Calabretta, B. (1989) *Science* **245**, 180–183.
8. Venturelli, D., Travali, S. & Calabretta, B. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5963–5967.
9. Travali, S., Reiss, K., Ferber, A., Mercer, W. E., Petralia, S., Calabretta, B. & Baserga, R. (1991) *Mol. Cell. Biol.* **11**, 731–736.
10. Nomura, N., Takahashi, M., Matsui, M., Ishii, S., Date, T., Shigemi, S. & Ishizaki, R. (1988) *Nucleic Acids Res.* **16**, 11075–11089.
11. Mizuguchi, G., Nakagoshi, H., Nagase, T., Nomura, N., Date, T., Ueno, Y. & Ishii, S. (1990) *J. Biol. Chem.* **265**, 9280–9287.
12. Golay, J., Capucci, A., Arsura, M., Castellano, M., Rizzo, V. & Introna, M. (1991) *Blood* **77**, 149–156.
13. Reiss, K., Travali, S., Calabretta, B. & Baserga, R. (1991) *J. Cell. Physiol.* **148**, 338–343.
14. Pledger, W. J., Stiles, C. D., Antoniades, H. N. & Scher, C. D. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4481–4485.
15. Stiles, C. D., Capone, G. T., Scher, C. D., Antoniades, H. N., Van Wyk, J. J. & Pledger, W. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1279–1283.
16. Matsushime, H., Roussel, M. F., Ashmum, R. A. & Scherr, C. J. (1991) *Cell* **65**, 701–710.
17. Gritz, L. & Davies, J. (1983) *Gene* **25**, 179–188.
18. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
19. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5201–5205.
20. Wang, J., Chenivesse, X., Henglein, B. & Brechot, C. (1990) *Nature (London)* **343**, 555–558.
21. Pines, J. & Hunter, T. (1989) *Cell* **58**, 833–846.
22. Lee, M. G. & Nurse, P. (1987) *Nature (London)* **327**, 31–34.
23. Tokunaga, K., Taniguchi, H., Shimizu, M. & Sakiyama, S. (1986) *Nucleic Acids Res.* **14**, 2829.
24. Hunter, T. & Pines, J. (1991) *Cell* **66**, 1071–1073.
25. Motokura, T., Bloom, T., Kim, H. G., Juppner, H., Ruderman, J. U., Kronenberg, H. M. & Arnold, A. (1991) *Nature (London)* **350**, 512–515.
26. Xiong, X., Connolly, T., Futcher, B. & Beach, D. (1991) *Cell* **65**, 691–700.
27. Lew, D. J., Dulic, V. & Reed, S. I. (1991) *Cell* **66**, 1197–1205.
28. Furukawa, Y., Piwnica-Worms, H., Ernst, T. J., Kanakura, Y. & Griffin, J. D. (1990) *Science* **250**, 805–808.
29. Withers, D. A., Harvey, R. C., Faust, J. B., Melnyk, O., Carey, K. & Meeker, T. C. (1991) *Mol. Cell. Biol.* **11**, 4846–4855.
30. Girard, F., Strausfeld, U., Fernandez, A. & Lamb, N. J. C. (1991) *Cell* **67**, 1169–1179.
31. D'Urso, G., Marracino, R. L., Marshak, D. R. & Roberts, J. M. (1990) *Science* **250**, 786–791.
32. Tsai, L.-H., Harlow, E. & Meyerson, M. (1991) *Nature (London)* **353**, 174–177.