

B-Myb Expression in Vascular Smooth Muscle Cells Occurs in a Cell Cycle-dependent Fashion and Down-regulates Promoter Activity of Type I Collagen Genes*

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The members of the Myb family of transcription factors are defined by homology in the DNA-binding domain; all bind the Myb-binding site (MBS) sequence (YG(A/G)C(A/C/G)GTT(G/A)). Here we report that cultured bovine vascular smooth muscle cells (SMCs) express B-*myb*. Levels of B-*myb* RNA found in exponential growth were reduced dramatically in serum-deprived quiescent SMCs; B-*myb* mRNA levels increased in the cell cycle during the late G₁ to S phase transition following restimulation with serum, epidermal growth factor, or phorbol ester plus insulin-like growth factor-1. Changes in the rate of B-*myb* gene transcription could account for part of the observed increase following serum addition. Treatment of SMC cultures with actinomycin D indicated a >4-h half-life for B-*myb* mRNA during the S phase of the cell cycle. Cotransfection of either a bovine or human B-*myb* expression vector down-regulated the activity of a multimerized MBS element-driven reporter construct in SMCs. Putative MBS elements were detected upstream of the promoters of the two chains of type I collagen, which we have found to be expressed inversely with growth state of the SMC (Kindy, M. S., Chang, C.-J., and Sonenshein, G. E. (1988) *J. Biol. Chem.* 263, 11426–11430). In cotransfection experiments, B-*myb* expression down-regulated the promoter activity of $\alpha 1(I)$ and $\alpha 2(I)$ collagen constructs an average of 92 and 82%, respectively. Thus, B-*myb* represents a potential link in the observed inverse relationship between collagen gene expression and growth of vascular SMCs.

The *myb* oncogene was first identified as the transforming gene of two retroviruses, avian myeloblastosis virus and E26, both of which cause myeloblastic leukemia in birds (Moscovici, 1975). In nontransformed cells, high levels of *c-myb* mRNA are observed only in immature hematopoietic cells (Gonda and Metcalf, 1984), while lower *myb* levels have been detected in embryonic neural tissue as well as neuroblastoma cells and in chick embryo fibroblasts (Thiele *et al.*, 1988; Thompson *et al.*, 1986). We found that cultured bovine vascular smooth muscle cells (SMCs)¹ also express low levels of *c-myb* mRNA (Reilly *et*

al., 1989; Brown *et al.*, 1992). Two *c-myb* related genes have been isolated based on their high homology in the DNA-binding domains (Nomura *et al.*, 1988; Lam *et al.*, 1992). These genes, termed A- and B-*myb*, have only recently begun to be characterized.

B-*myb* expression has been detected in many tissues (Nomura *et al.*, 1988; Golay *et al.*, 1991; Arsura *et al.*, 1992, 1994). Cell synchronization studies have demonstrated that in 3T3 fibroblasts and hematopoietic cells, B-*myb* displays a late G₁-specific gene expression pattern, similar to that of *c-myb* (Lam *et al.*, 1992; Golay *et al.*, 1991; Reiss *et al.*, 1991). Recent work has indicated that B-Myb protein is capable of binding to the consensus Myb-binding site (MBS) (YGRC(A/C/G)GTT(G/A)) (Howe and Watson, 1991), although R is preferably T/C for c-Myb and A/C for B-Myb. Furthermore, B-Myb has also been reported to recognize a second specific consensus sequence (CUNTTTCT) as well (Mizuguchi *et al.*, 1990). The transactivation properties of B-*myb* are controversial, as one group reported it to function as a positive regulator (Mizuguchi *et al.*, 1990), while several others had found it to be a transcriptional inhibitor of *c-myb*-mediated transactivation (Foos *et al.*, 1992; Watson *et al.*, 1993). These apparently contradictory findings may be due to the fact that transfected B-*myb* behaves differently in different cell lines (Tashiro *et al.*, 1995). B-*myb* inhibited *c-myb*-induced transactivation in 3T3 fibroblasts, whereas activation was observed upon transfection into HeLa cells. Although no mechanism has been established for this effect, Tashiro *et al.* (1995) proposed that cell-specific expression of binding partner proteins allows for differential formation of a functional dimer. In addition, B-*myb* has also been found to transactivate the DNA polymerase α gene promoter independent of any identified MBS element (Venturelli *et al.*, 1990; Watson *et al.*, 1993).

SMCs are the major cellular constituents of the medial layer of an artery, being responsible for maintaining vascular tone in the adult blood vessel (Ross, 1993). During the formation of a developing artery, SMCs display a synthetic phenotype; an initial highly proliferative phase is followed by synthesis of extracellular matrix components such as collagen, elastin, and proteoglycans (Hughes, 1942; Wu *et al.*, 1992). This matrix provides a structural framework for the artery and also presumably allows for cell layering. Once the artery has been fully formed, SMCs differentiate into a contractile phenotype, in which they normally remain (Chamley-Campbell *et al.*, 1979). As a normal response to injury and in certain disease states, however, SMCs migrate to the intimal layer, where modest rounds of proliferation are followed by production and deposition of matrix components over extended periods of time (Poole

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¹ The abbreviations used are: SMCs, smooth muscle cells; MBS, Myb-binding site; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EGF, epidermal growth factor; TPA, 12-O-tetradecano-

ylphorbol-13-acetate; IGF-1, insulin-like growth factor-1; kb, kilobase(s); bp, base pair(s); CAT, chloramphenicol acetyltransferase; TK, thymidine kinase.

et al., 1971; Schwartz *et al.*, 1985; Gordon *et al.*, 1990; Ross, 1993; Strauss *et al.*, 1994). These synthetic responses of SMCs, in association with deposition of lipids and minerals, can result in formation of an atherosclerotic plaque.

SMCs grown in culture maintain a dedifferentiated synthetic phenotype. At low cell density, they proliferate rapidly, but produce little connective tissue matrix (Beldekas *et al.*, 1982; Stepp *et al.*, 1986). As we and others have shown, production of connective tissue proteins, such as collagen types I, III, and V, by SMCs increases dramatically as they approach confluence, when their growth slows and cells begin to form multilayers (Beldekas *et al.*, 1982; Liao and Chan, 1989; Ang *et al.*, 1990; Brown *et al.*, 1991). Since vascular SMCs express the *c-myc* oncogene, here we characterized expression of B-*myb*. Aortic SMCs were found to express B-*myb* in a cell cycle-dependent fashion; quiescent cells contained low levels of B-*myb* RNA, with increasing levels seen during the late G₁ to S phase transition. Cotransfection of B-*myb* expression vectors in SMC cultures inhibited the activity of a multimerized MBS-driven heterologous promoter reporter construct and of the promoters of the $\alpha 1(I)$ and $\alpha 2(I)$ collagen genes, which contain putative elements for B-Myb binding. These findings suggest that B-*myb*, which is expressed at high levels in growing cells, may play a role in down-regulating collagen gene expression in proliferating SMCs.

MATERIALS AND METHODS

Cell Culture and Treatment Conditions—Smooth muscle cell explants were derived from the aortas of female calves, as we have described previously (Beldekas *et al.*, 1982). Cultures were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1% sodium pyruvate, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies, Inc.). The medium was changed every 2–3 days, and cells were not used beyond the fourth passage. SMC cultures were synchronized as described previously (Kindy and Sonenshein, 1986). Briefly, cells were plated at low density (5×10^5 cells/150-mm² dish) and allowed to grow exponentially for 3 days, at which time the medium was changed to DMEM supplemented with 0.5% FBS. Cells were maintained in 0.5% FBS for 3 days to achieve quiescence, at which time the cells were then stimulated with fresh DMEM containing 10% FBS. With this procedure (Kindy and Sonenshein, 1986), we have found that only 1–2% of SMCs deprived of serum for 72 h demonstrate significant [³H]thymidine nuclear labeling. Serum stimulation results in an increase in labeled nuclei at 12 h, indicating the beginning of DNA synthesis, with percent nuclear labeling increasing to 95% after 20 h of serum stimulation (Kindy and Sonenshein, 1986; Brown *et al.*, 1992). Levels of histone H3.2 mRNA, an S phase-specific gene, and cytofluorometric measurements further confirmed cell synchrony (Kindy and Sonenshein, 1986). Alternatively, serum-deprived cells were stimulated with either 20 ng/ml EGF or 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the absence or presence of 35 ng/ml insulin-like growth factor-1 (IGF-1).

DNA Constructs—cDNA constructs employed were as follows: $\alpha 1(I)$ collagen, pCg54, a chicken cDNA clone (Lehrach *et al.*, 1979); $\alpha 1(V)$ collagen, pTV302, a human cDNA clone (Takahara *et al.*, 1991); B-*myb*, λ -B-*myb*, a human cDNA clone (Nomura *et al.*, 1988); histone H3.2, pRAH3.2, a genomic fragment encoding amino acids 57–125 of histone H3.2 (Alterman *et al.*, 1984); and ornithine decarboxylase, murine cDNA clone pOD48 (McConlogue *et al.*, 1984).

A bovine B-*myb* cDNA plasmid expression vector, pB14, was isolated from a custom cDNA library, constructed by Stratagene (La Jolla, CA), in λ -ZAP-EXPRESS using poly(A⁺) RNA from exponentially growing aortic SMCs. The 3.4-kb insert of pB14 was the largest isolated in the screening and represents a nearly full-length cDNA based on the 3.5-kb B-*myb* mRNA size estimate. Partial DNA sequence information of a *Pst*I fragment of pB14 subcloned into the Bluescript vector, obtained by double-stranded sequencing with Sequenase Version 2.0 (U.S. Biochemical Corp.), indicated homology of >84% to the human B-*myb* sequences (bp 1755–1941) (Nomura *et al.*, 1988), analyzed using the Blast program (National Center for Biotechnology Information). The human B-*myb* expression vector pCEP-B-*myb* contains the *Bam*HI fragment, including the entire coding region of the B-*myb* gene from plasmid pATB-18 (Arsura *et al.*, 1992), subcloned into the pCEP4 β plasmid

expression vector (Invitrogen).

The reporter plasmid KHK-CAT-dAX was derived by insertion of nine copies of the MBS directly in front of the thymidine kinase promoter linked to the chloramphenicol acetyltransferase gene in dAX-TK-CAT (Ibanez and Lipsick, 1990). The vector dAX-TK-CAT was in turn constructed from pBLCAT2 by deletion of the *Aat*II polylinker (*Xho*I) fragment from the pUC18 plasmid backbone, which appeared to confer a low level of *myb*-induced transcription activity apparently caused by cryptic MBS elements (Ibanez and Lipsick, 1990). The vector p1.6Bgl-CAT contains bp –1114 to +513 of the murine *c-myc* gene linked to the CAT reporter construct as described previously (Duyao *et al.*, 1992). pHNmyb-CAT contains 1 kb of sequence upstream of the start site of transcription of the human *c-myc* promoter and 1.1 kb of exon 1 cloned into the pSV₀CAT vector (kindly provided by T. Bender, University of Virginia School of Medicine, Charlottesville, VA). The pMS-3.5/CAT construct contains bp –3500 to +58 of the human $\alpha 2(I)$ promoter upstream of the CAT reporter gene (Boast *et al.*, 1990). The plasmid pOB3.6 contains 3.5 kb of the rat $\alpha 1(I)$ collagen promoter plus the first exon and first intron linked to the CAT reporter (Bedalov *et al.*, 1994). The plasmid ColCAT3.6 is composed of a 3.6-kb fragment containing 3.5 kb of sequence upstream of the start site of transcription and 115 bp of the first exon of the rat $\alpha 1(I)$ collagen gene linked to the CAT reporter (Lichtler *et al.*, 1989).

Transfections and Reporter Gene Assays—Cells were plated at a density of 5×10^5 cells/100-mm² dish 24 h before transfection. The medium was changed 2–4 h before transfection. DNA (50 μ g) was transfected by the modified CaPO₄ transfection procedure of Chen and Okayama (1987). Cells were harvested 48–72 h after transfection, and lysates were prepared as described previously (Lawrence *et al.*, 1994a). Protein concentrations of the lysates were determined using the Bradford assay as directed by the manufacturer (Bio-Rad). Equal amounts of total protein were incubated with 2.5 μ Ci of [³H]acetyl coenzyme A (DuPont NEN; 200 mCi/mmol), 50 μ M acetyl coenzyme A, and 1.6 mM chloramphenicol for 4–8 h, and the acetylated forms were extracted with ethyl acetate and assayed by liquid scintillation counting (Lawrence *et al.*, 1994a).

RNA Isolation and Hybridization Analysis—Total cellular RNA was isolated according to the method of Chirgwin *et al.* (1979) or with Tri-Reagent (Molecular Research Center, Inc.). Equal quantities of RNA (15–25 μ g) were denatured and separated by electrophoresis on 1.0% agarose-formaldehyde gels. Separated RNA was transferred onto a GeneScreen Plus nylon membrane (DuPont NEN). RNA was cross-linked to the membrane by UV irradiation (Stratalinker, Stratagene) at 0.12 J/cm² for 30 s. For RNA stability studies, cells were treated with 5 μ g/ml actinomycin D (Boehringer Mannheim). Probes were prepared as described previously by Feinberg and Vogelstein (1982); hybridization reactions contained 1–2 $\times 10^6$ cpm of ³²P-labeled DNA/ml of buffer. Unhybridized probe was removed by washing blots at 68 °C with 2 \times SSC (1 \times SSC is 0.15 M NaCl, 15 mM sodium citrate), 0.1% SDS for 30 min, followed by 15–30 min washes with 1 and 0.5 \times SSC, as needed. Quantitation by scanning densitometry was performed using a Molecular Dynamics 300A computing densitometer.

Transcription Analysis—Nuclei were isolated from SMCs, and run-off analysis was performed by a modification of the method of Greenberg and Ziff (1984). Briefly, $\sim 1 \times 10^7$ nuclei were incubated in the presence of 250 μ Ci of [³²P]UTP (DuPont NEN; 3200 Ci/mmol) for 30 min at 30 °C. Labeled RNA was isolated, and equal amounts of radio-labeled RNA (4.5×10^6 cpm/ml of hybridization buffer) were hybridized to plasmid DNA (10 μ g/sample) immobilized onto GeneScreen Plus by slot blotting followed by UV irradiation; after hybridization, blots were washed as described above.

RESULTS

Expression of the B-*myb* mRNA in Aortic SMC Cultures—Expression of B-*myb* in hematopoietic cells and 3T3 fibroblasts has been shown to be cell cycle-regulated, with increasing levels detected during the G₁ to S phase transition (Golay *et al.*, 1991; Lam *et al.*, 1992). To determine whether aortic SMCs express B-*myb*, Northern blot analysis was performed using RNA isolated from bovine aortic SMCs synchronized using the serum deprivation-stimulation protocol described previously (Kindy and Sonenshein, 1986; see “Materials and Methods”). Serum-deprived quiescent cells begin to enter S phase ~ 12 h after serum stimulation, and DNA synthesis peaks between 16 and 20 h (Kindy and Sonenshein, 1986). RNA was isolated from SMCs in exponential growth, in quiescence, and at various

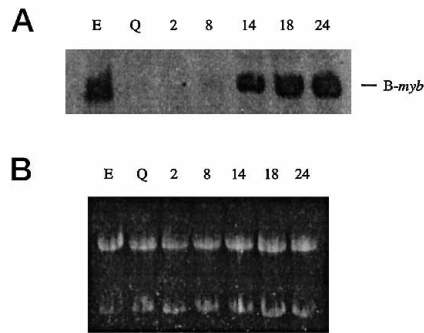


FIG. 1. Cell cycle expression of B-*myb* RNA in bovine vascular smooth muscle cells. Bovine aortic SMC cultures were rendered quiescent by serum deprivation (DMEM plus 0.5% FBS) for 72 h. Serum was then added back (DMEM plus 10% FBS) to allow synchronous entry into S phase. Total RNA, isolated at the indicated time points and from cells in exponential growth and quiescence, was subjected to Northern blot analysis. A, autoradiogram of a blot probed with the human λ -B-*myb* cDNA clone; B, ethidium bromide-stained gel confirming RNA quality and equal loading. Lane E, exponential growth; lane Q, quiescence; lanes 2, 8, 14, 18, and 24, numbers indicate the hours after serum addition.

times following serum restimulation. Using a human B-*myb* cDNA clone as a probe, Northern blot analysis revealed that exponentially growing SMCs express significant quantities of an ~3.5-kb B-*myb* mRNA (Fig. 1). In contrast, very low levels of B-*myb* RNA were seen in quiescent SMCs, and RNA levels remained low until 8 h after serum stimulation. Between 8 and 14 h of serum stimulation, levels of B-*myb* RNA began to increase and continued to do so until 24 h after serum stimulation. The level of B-*myb* mRNA increased ~6–8-fold during this time as judged by densitometric scanning. Entry into S phase between 12 and 16 h was verified using incorporation of [3 H]thymidine and appearance of histone H3.2 mRNA as described previously (Kindy and Sonenshein, 1986) (data not shown). Therefore, B-*myb* is expressed in bovine aortic SMCs in a cell cycle-dependent manner. B-*myb* RNA levels are low in quiescence, increase in the mid to late G₁ phase of the cell cycle, immediately prior to the onset of DNA synthesis, and continue to increase throughout S phase.

Regulation of B-*myb* Gene Transcription—To determine whether transcriptional control mechanisms play a role in the increase in steady-state B-*myb* RNA levels, nuclear run-off analysis was performed. Nuclei were isolated from cells shortly after serum stimulation (0.5 h), to allow adequate time for protein synthesis to recover from the low levels in quiescent cells, and at 12 and 18 h after serum stimulation, to compare transcription rates in late G₁ and mid S phase. Hybridization to the B-*myb* probe increased significantly between 0.5 and 12 h and remained constant between 12 and 18 h (Fig. 2). In two separate experiments, an ~1.6–2-fold increase was seen. In contrast, transcription of the α 1 chains of collagen types I and V decreased between 12 and 18 h and between 0.5 and 12 h, respectively. Transcription of ornithine decarboxylase and histone H3.2 increased by 12 h, as expected (McConlogue *et al.*, 1984; Kindy and Sonenshein, 1986). These results indicate that there is an increase in the rate of B-*myb* gene transcription during the cell cycle; this increase can account for a part of the increase in steady-state RNA levels seen by Northern analysis.

B-*myb* mRNA Decays Slowly—To assess the half-life of B-*myb* mRNA, the rate of decay of B-*myb* RNA was determined following addition of the transcriptional inhibitor actinomycin D to SMCs in S phase. At 18 h after serum stimulation, cells were treated for 1–4 h with 5 μ g/ml actinomycin D, which we have found effectively inhibits RNA synthesis (Kindy and Sonenshein, 1986). No decay of B-*myb* RNA was observed even

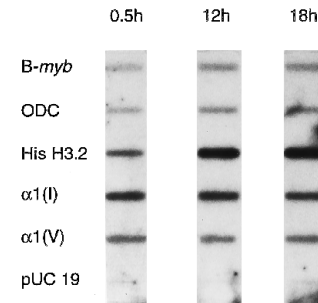


FIG. 2. Nuclear run-off analysis of cell cycle changes in the rate of transcription of the B-*myb* gene. SMC cultures were made quiescent and serum-stimulated as described in the legend to Fig. 1. Nuclei were isolated at 0.5, 12, and 18 h after serum addition and subjected to nuclear run-off analysis. The resulting radiolabeled RNAs were hybridized to the following cDNA probes (10 μ g) immobilized on nylon membranes: bovine B-*myb* (pB14), ornithine decarboxylase (ODC), histone H3.2 (His H3.2), α 1(I) collagen, α 1(V) collagen, and pUC19 plasmid DNA.

after 4 h of actinomycin D treatment (Fig. 3). In contrast, levels of the S phase-expressed histone H3.2 mRNA, which were significant at 18 h, decayed substantially during this treatment, consistent with results obtained previously (Brown *et al.*, 1992). Therefore, B-*myb* mRNA appears relatively stable at the time points where substantial steady-state levels are present.

Activation of B-*myb* by EGF and TPA plus IGF-1—We next investigated the ability of specific growth factors to induce expression of B-*myb* RNA. Subconfluent SMC cultures were made quiescent via serum deprivation and were then stimulated with addition of 20 ng/ml EGF. This growth factor is a weak mitogen for subconfluent SMCs, as evidenced by the appearance of histone H3.2 mRNA, which was detectable by 24 h (Fig. 4). EGF treatment resulted in a low level of induction of B-*myb* mRNA that began to be detectable at 12 h and was clearly seen at 24 h (Fig. 4). Thus, expression of B-*myb* RNA was inducible by EGF and appeared to precede entry into S phase, consistent with the G₁/S-phase specific nature of the B-*myb* expression observed above.

Phorbol ester treatment of quiescent cells has been found to induce genes mediating competence, such as *c-fos* and *c-myc*, and entry into the G₁ phase of the cell cycle (Greenberg and Ziff, 1984; Kelly *et al.*, 1983). Further transit from G₁ into S phase requires stimulation with a progression factor, such as IGF-1 (Leof *et al.*, 1982). To examine the effects of these agents on SMCs, serum-deprived quiescent cell cultures were stimulated with 100 nM TPA in the absence or presence of 35 ng/ml IGF-1. RNA was isolated from cells in quiescence (0 h) or 10, 16, and 24 h after stimulation. B-*myb* RNA levels were low in quiescence (Fig. 5), as observed above (Fig. 1). No significant increase in B-*myb* expression was seen with TPA treatment alone. In contrast, B-*myb* RNA levels increased in the cells treated with both TPA and IGF-1. Thus, treatment with TPA made SMCs competent to respond to the progression factor IGF-1, leading to increased expression of B-*myb*.

Activity of B-*myb* as a Transcriptional Regulator—Previously, we had shown that the reporter plasmid KHK-CAT-dAX, derived by insertion of nine copies of the MBS directly in front of the TK promoter linked to the CAT gene in the plasmid dAX-TK-CAT, was transcriptionally active in SMCs (Brown *et al.*, 1992). To begin to assess the specific functional role of B-*myb* in transactivational control in the vascular SMC, a 3.4-kb bovine pB14 B-*myb* cDNA clone, in the pBK-CMV plasmid expression vector, was isolated from an aortic SMC cDNA library and used in cotransfection experiments. SMC cultures, at ~50% confluence, were cotransfected with the KHK-CAT-

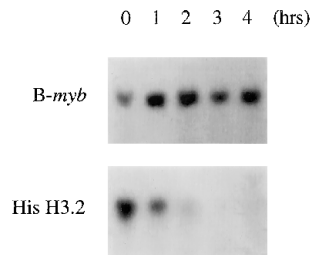


FIG. 3. Decay of mRNA following actinomycin D treatment in the S phase of the cell cycle. SMC cultures were made quiescent and serum-stimulated as described in the legend to Fig. 1. At 18 h following serum addition, 5 μ g/ml actinomycin D was added; total RNA was isolated after 1, 2, 3, and 4 h; and samples (15 μ g) were subjected to Northern blot analysis for B-myb (pB14 cDNA clone) and histone H3.2 (*His H3.2*) mRNAs. RNA integrity and equal loading were confirmed by staining with ethidium bromide.

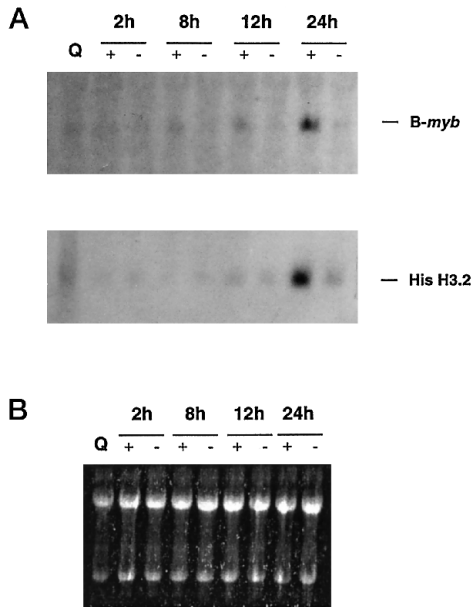


FIG. 4. Effects of EGF on expression of B-myb RNA in bovine vascular SMCs. Bovine aortic SMC cultures were rendered quiescent by serum deprivation (DMEM plus 0.5% FBS) for 72 h. EGF (20 ng/ml) was added, and total RNA was isolated at the indicated time points and from cells in quiescence (Q) and subjected to Northern blot analysis. A, autoradiogram of a blot probed with human λ -B-myb cDNA and histone H3.2 (*His H3.2*) genomic clones, respectively; B, ethidium bromide-stained gel confirming RNA quality and equal loading.

dAX reporter construct and increasing amounts of pB14 expression vector. After 3 days, extracts were prepared, and equal amounts of proteins were analyzed. B-myb expression resulted in dose-dependent down-regulation of KHK-CAT-dAX activity, with a maximal decrease of ~ 2.5 -fold or 60% (Fig. 6). An average of three separate experiments yielded a drop in activity of $60.3 \pm 3.7\%$. In contrast, pB14 had no effect on the activity of parental dAX-TK-CAT. Cotransfection with 12 μ g of pB14 only reduced dAX-TK-CAT activity to $92.0 \pm 4.0\%$ of control in two separate experiments (data not shown). Cotransfection of 5 μ g of a human pCEP-B-myb expression vector similarly resulted in a specific down-regulation of the activity of KHK-CAT-dAX (65%; data not shown). Thus, expression of B-Myb leads to repression of the transcriptional activity of an MBS element-driven heterologous promoter in SMCs.

Regulation of Collagen Promoters by B-myb—Previously, we had noted that type I collagen mRNA levels varied inversely with the growth state of the vascular SMC (Stepp *et al.*, 1986; Kindy *et al.*, 1988). Levels of type I collagen mRNA were low in actively proliferating SMCs and increased in quiescence due to

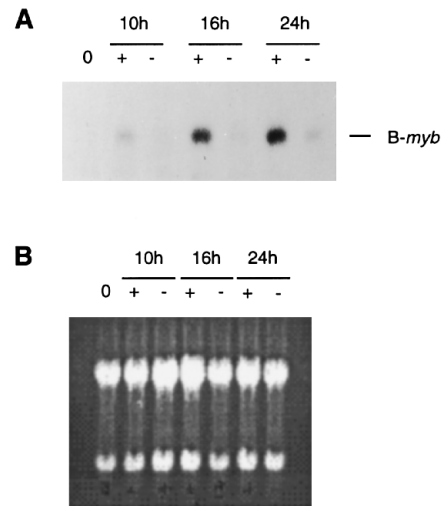


FIG. 5. Effects of TPA in combination with IGF-1 on expression of B-myb RNA in bovine vascular SMCs. SMC cultures were rendered quiescent by serum deprivation (DMEM plus 0.5% FBS) for 72 h. Cells were treated with 100 nM TPA in the absence (–) or presence (+) of 35 ng/ml IGF-1. Total RNA was isolated at the indicated time points as well as from cells in quiescence (0 h) and subjected to Northern blot analysis. A, autoradiogram of a blot probed with the human B-myb cDNA clone probe; B, ethidium bromide-stained gel confirming RNA quality and equal loading.

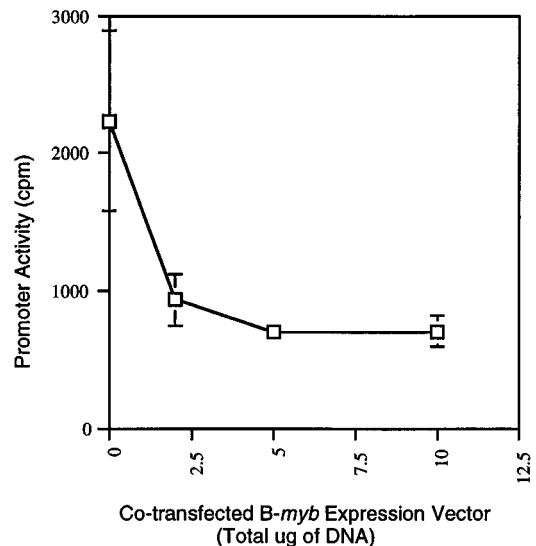


FIG. 6. Activity of B-myb as a transcriptional regulator of an MBS element-driven construct in SMCs. Twenty-five micrograms of the reporter plasmid KHK-CAT, containing nine MBS elements upstream of the TK promoter and CAT gene, were cotransfected in duplicate into aortic SMCs with increasing amounts of bovine B-myb expression vector pB14. pUC19 DNA was used to equalize the total amounts of DNA transfected (50 μ g/100-mm² dish).

either serum deprivation or growth to confluence. Since bovine B-myb appeared to be a negative regulator of transcription, we analyzed the promoters of the type I collagen genes and identified several putative MBS elements in both the $\alpha 1$ and $\alpha 2$ chains of type I collagen (see "Discussion"). Thus, cotransfection experiments were performed to test the effects of B-myb expression on collagen promoter activity. Increasing concentrations of pB14 were cotransfected with the $\alpha 2(I)$ collagen promoter pMS-3.5/CAT construct, which contains 3.5 kb of sequence upstream of the start site of transcription and 58 bp of exon 1 driving the CAT reporter gene (Boast *et al.*, 1990). The activity of the pMS-3.5/CAT vector was down-regulated 3.8-fold (72%) upon cotransfection with 10 μ g of bovine B-myb vector

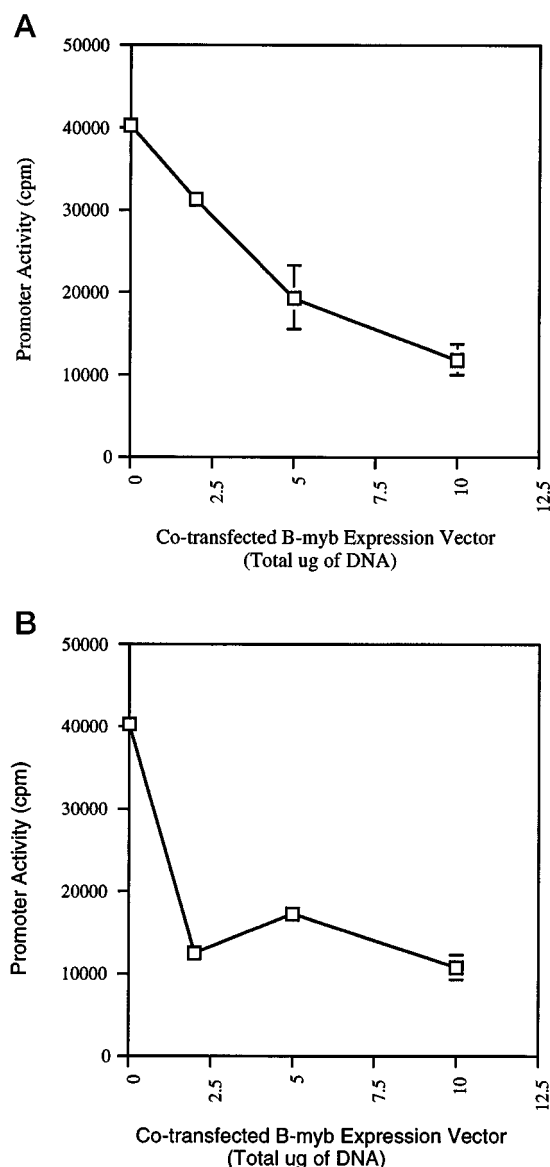


FIG. 7. Effects of B-myb expression on the activity of the $\alpha 2(I)$ collagen promoter in SMCs. SMCs were transfected in duplicate with 25 μ g of pMS-3.5/CAT collagen $\alpha 2(I)$ promoter reporter construct (Boast *et al.*, 1990) in the presence of the indicated amounts of B-myb expression vector and of pUC19 DNA to make up a total of 50 μ g of DNA/100-mm² dish. Extracts containing equal amounts of protein were assayed for CAT activity. A, bovine B-myb; B, human B-myb.

(Fig. 7A). An average decrease of $82 \pm 10.8\%$ was noted in three experiments upon cotransfection with 10 μ g of pB14 DNA. Cotransfection with a human B-myb expression vector similarly down-regulated the activity of the $\alpha 2(I)$ promoter (Fig. 7B). The significance of the differences in the slopes of the two curves is unclear given the differences in the expression vectors used. An ~ 4 -fold (73%) down-regulation was observed with 2.5 μ g of human B-myb expression vector. Thus, B-myb, either bovine or human, inhibits the activity of the $\alpha 2(I)$ collagen promoter in SMCs.

The expression of the $\alpha 1$ and $\alpha 2$ genes of type I collagen is often coordinately regulated (Stepp *et al.*, 1986). Therefore, we assessed the effects of B-Myb expression on the activity of the $\alpha 1(I)$ promoter. pOB3.6, which contains 3.6 kb of the $\alpha 1(I)$ collagen promoter plus all of exon 1 and intron 1 upstream of the CAT reporter gene, was cotransfected with the bovine B-myb expression vector pB14. The activity of pOB3.6 was down-regulated ~ 8.8 -fold, in a dose-dependent manner, by coex-

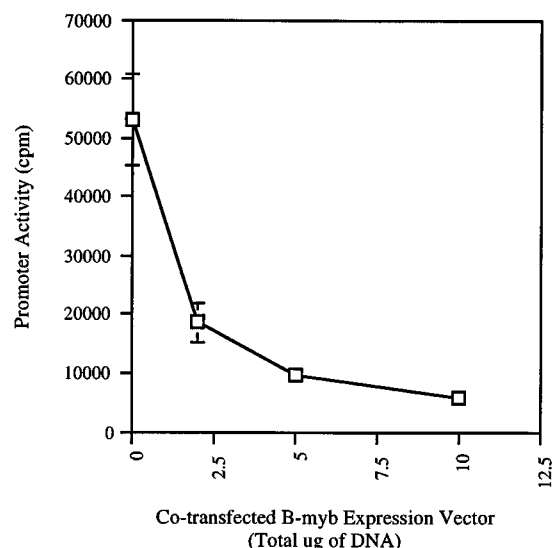


FIG. 8. Effects of bovine B-myb expression on the activity of the $\alpha 1(I)$ collagen promoter in SMCs. SMCs were transfected in duplicate with 25 μ g of pOB3.6 collagen $\alpha 1(I)$ promoter reporter construct (Bedalov *et al.*, 1994) in the presence of the indicated amounts of bovine B-myb expression vector and of pUC19 DNA to make up a total of 50 μ g of DNA/100 mm² dish. Extracts containing equal amounts of protein were assayed for CAT activity.

sion of B-myb (Fig. 8). Cotransfection with 10 μ g of bovine pB14 reduced pOB3.6 activity an average of $92 \pm 2.5\%$ in three experiments, and that with 10 μ g of human pCEP-B-myb expression vector reduced it 79% (data not shown). The plasmid ColCAT3.6, which contains 3.5 kb of the $\alpha 1(I)$ collagen promoter plus 115 bp of exon 1 upstream of the CAT reporter gene, displayed fairly low levels of CAT activity in SMCs (data not shown). This activity was similarly down-regulated by the presence of either human or bovine B-myb, but to a somewhat lesser extent, $57 \pm 2.4\%$ (data not shown). Therefore, B-Myb is a specific regulator of transcription that is able to down-regulate the activity of the promoters of the genes encoding both chains of type I collagen.

To determine whether B-myb acts nonspecifically as a negative regulator of transcription in SMCs, cotransfection analysis was performed with the *c-myc* and *c-myb* promoters, both of which contain MBSs that have been shown to be regulated by c-Myb (Evans *et al.*, 1990; Nakagoshi *et al.*, 1992; Nicolaides *et al.*, 1991). Cotransfection of 5 μ g of pB14 with the *c-myc* promoter plasmid p1.6Bgl-CAT resulted in only an $\sim 15\%$ reduction in its activity. These results agree with those of Watson *et al.* (1993), who found that B-myb had no effect on *c-myc* promoter activity in 3T3 fibroblasts. Similarly, the activity of the *c-myb* promoter plasmid pHNmyb-CAT was down-regulated only 12% upon cotransfection with B-myb. Thus, B-myb expression did not appear to significantly affect the promoter activity of these two oncogenes, suggesting that the inhibition of collagen promoter activity described above is specific.

DISCUSSION

Proliferating primary bovine aortic SMCs were found to express B-myb, a member of the myb gene family. B-myb expression in SMCs occurred in a cell cycle-dependent fashion and displayed negative regulatory activity with respect to an MBS element-driven construct and the $\alpha 1(I)$ and $\alpha 2(I)$ collagen promoters. Quiescent SMCs expressed very little B-myb mRNA, and levels increased as cells entered late G₁ and peaked in S phase following stimulation with serum, EGF, or a combination of treatment with TPA and IGF-1. Previous work in several laboratories, including our own, demonstrated an inverse rela-

tionship between SMC growth and collagen production (Jones *et al.*, 1979; Stepp *et al.*, 1986; Kindy *et al.*, 1988; Liau and Chan, 1989; Ang *et al.*, 1990; Chang and Sonenshein, 1991). When SMCs were proliferating rapidly, collagen gene expression was low, whereas when they were confluent or made quiescent via either serum starvation or isoleucine deprivation, collagen mRNA expression increased significantly. The findings presented here suggest the intriguing possibility that B-Myb mediates signals regulating this inverse relationship between growth and collagen gene expression in SMCs; furthermore, they indicate that collagen genes represent a new family of targets for regulation by a member of the *myb* gene family.

When transfected into aortic SMCs, bovine B-*myb* negatively regulated an MBS element/heterologous promoter-driven construct. Thus, the overexpression of B-*myb* can apparently override, presumably via competition for binding, the induction of MBS element activity by the low level of endogenously expressed *c-myb* previously noted in these cells (Brown *et al.*, 1992). This is similar to results obtained in other cell types with the human and murine homologs of B-*myb*. In 3T3 cells, for example, murine B-*myb* has been shown to be a competitive inhibitor of *c-myb*-induced transactivation of an MBS-driven construct (Watson *et al.*, 1993). GAL4 fusion studies have also shown that the C terminus of murine B-Myb has no intrinsic ability to transactivate when fused with the DNA-binding domain of the GAL4 protein. This lack of transactivating ability is not due simply to lack of DNA binding by B-Myb since gel shift and footprinting studies have shown that B-Myb was able to bind to an MBS. Recently, it has been shown that B-Myb is able to function as a strong transcriptional activator when transfected into certain cell types, such as HeLa cells (Tashiro *et al.*, 1995). It has been postulated that this cell type specificity relates to the absence or presence of a cofactor that binds to B-Myb in its C-terminal conserved region and mediates transactivation.

The selective down-regulation of the activity of the promoters for the two chains of type I collagen upon B-*myb* expression in transient cotransfection analysis in primary cultured SMCs may occur by either a direct or an indirect mechanism. DNA analysis of the rat and human collagen COL1A1 and A2 genes revealed the presence of several putative Myb-binding sites and B-Myb-specific sequence elements. The presence of these putative sites raises the possibility of a direct effect of B-Myb on collagen genes. The limited size of these sequences, however, necessitates more specific mapping analysis. It should also be noted that indirect mechanisms have been observed, *e.g.* with the DNA polymerase α promoter (Venturelli *et al.*, 1990; Watson *et al.*, 1993); these could similarly be involved with the down-regulation of transcription of collagen genes via expression of B-Myb and would be of equal functional significance for matrix formation by the SMC. We have recently found that co-microinjection of B-*myb* with *c-myc* expression vectors into quiescent SMCs failed to induce entry into S phase, suggesting that the observed inhibition of collagen gene transcription is not simply due to a change in the proliferative state of the cell.²

The 6–8-fold increase in B-*myb* mRNA seen in the cell cycle can be partly accounted for by the 1.6–2-fold increase in the overall rate of transcription of the gene. In 3T3 fibroblasts, the mechanism of the cell cycle increase in B-*myb* RNA levels was determined to be due to an increase in the rate of transcription of the gene. Deletion of an E2F site abrogated cell cycle regu-

lation of B-*myb* transcription (Lam and Watson, 1993). Gel shift analysis revealed that quiescent cells showed E2F binding that was supershifted only with antibodies to E2F and p107, while in S phase, this complex contained cyclin A as well. It is possible that other mechanisms play a significant role in B-*myb* expression in SMCs. Alternative levels of control include either a change in the rate of elongation of RNA chains during synthesis or of RNA processing or altered stability. For example, *c-myb* mRNA levels in hematopoietic cells are controlled mainly by the rate of elongation of transcription (Bender *et al.*, 1987), while in chick embryo fibroblasts, mRNA stability is the main level of regulation (Thompson *et al.*, 1986). Interestingly, the increase in *c-myb* mRNA levels in SMCs during the late G₁ to S phase transition could not be accounted for either by an enhanced rate of gene transcription or by a change in the stability of the *c-myb* RNA (Brown *et al.*, 1992), suggesting additional levels of control.

The observation that there is an inverse relationship between matrix deposition and cellular proliferation is a long standing one that has been substantiated in many different systems. For example, viral transformation of fibroblasts enhanced the proliferative capacity of these cells while decreasing their level of synthesis of type I collagen (Adams *et al.*, 1982). Overexpression of the *ras* oncogene in Rat1 fibroblasts had a similar affect on type I collagen gene expression by these cells (Slack *et al.*, 1992). In density-arrested nondividing human fetal lung fibroblasts, type I and III collagen mRNA levels were significantly higher than those in logarithmically growing cells (Miskulin *et al.*, 1986). Thus, it appears that when genes necessary for growth, such as oncogenes, are expressed, other genes that are inconsistent with or unnecessary for growth are turned off. The fact that B-*myb* is expressed broadly in many different cell types presents the possibility that the signal transduction pathway that mediates activation of this gene may be involved in the inhibition of collagen gene expression in cells derived from many different tissues.

SMCs are responsible for synthesizing the extracellular matrix components in the medial layer of a normal artery, including collagen, elastin, fibronectin, and proteoglycans, as well as the enzymes involved in matrix protein deposition, such as lysyl oxidase (reviewed by Ross (1993)). During arterial development in the chick, an initial SMC synthetic phase is followed by deposition of matrix proteins and additional cell layering (Hughes, 1942; Wu *et al.*, 1992). The most abundant collagen species produced by SMCs is type I collagen, with lesser but still significant amounts of collagen types III, V, and VI. In atherosclerosis, SMCs migrate from the medial layer to the intima, where some initial rounds of proliferation are followed by extensive synthesis and deposition of matrix proteins (Poole *et al.*, 1971; Gordon *et al.*, 1990; Ross, 1993; Strauss *et al.*, 1994). The majority of the mass of a fibrous plaque is composed of the collagen proteins deposited by the SMC. The subsequent occlusion of the lumen of the artery and the clinical sequelae that follow are a primary cause of morbidity and mortality in the Western world. Coordinate regulation of many collagen species, including types I, III, and V, has been noted in SMC cultures under a variety of conditions that affect growth state (Jones *et al.*, 1979; Stepp *et al.*, 1986; Liau and Chan, 1989; Ang *et al.*, 1990; Brown *et al.*, 1991; Lawrence *et al.*, 1994b). In addition, other genes necessary for matrix deposition, such as lysyl oxidase, have similar inverse expression patterns in relation to growth (Kenyon *et al.*, 1991). Thus, the possibility that B-Myb plays a more general role in regulation of matrix gene expression in SMCs is under investigation.

² D. Marhamati, R. Bellas, M. Arsura, and G. E. Sonenshein, manuscript in preparation.

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REFERENCES

- Adams, S. L., Boettiger, D., Focht, R., Holtzer, H., and Pacifici, M. (1982) *Cell* **30**, 373–384
- Alterman, R., Ganguly, S., Schulze, D., Marzluff, W., Schildkraut, C., and Skoultschi, A. (1984) *Mol. Cell. Biol.* **4**, 123–132
- Ang, A. H., Tachas, G., Campbell, J. H., Bateman, J., and Campbell, G. R. (1990) *Biochem. J.* **265**, 461–469
- Arsura, M., Introna, M., Passerini, F., Mantovani, A., and Golay, J. (1992) *Blood* **79**, 2708–2716
- Arsura, M., Luchetti, M., Erba, E., Golay, J., Rambaldi, A., and Introna, M. (1994) *Blood* **83**, 1778–1790
- Bedalov, A., Breault, D., Sokolov, B., Lichtler, A., Bedalov, I., Clark, S., Mack, K., Khillan, J., Woody, C., Kream, B., and Rowe, D. (1994) *J. Biol. Chem.* **269**, 4903–4909
- Beldekis, J., Gerstenfeld, L., Sonenshein, G. E., and Franzblau, C. (1982) *J. Biol. Chem.* **257**, 12252–12256
- Bender, T., Thompson, C. B., and Kuehl, W. M. (1987) *Science* **237**, 1473–1476
- Boast, S., Su, S.-W., Ramirez, F., Sanchez, M., and Avvedimento, E. (1990) *J. Biol. Chem.* **265**, 13351–13356
- Brown, K. E., Lawrence, R., and Sonenshein, G. E. (1991) *J. Biol. Chem.* **266**, 23268–23273
- Brown, K. E., Kindy, M., and Sonenshein, G. E. (1992) *J. Biol. Chem.* **267**, 4625–4630
- Chamley-Campbell, J., Campbell, G. R., and Ross, R. (1979) *Physiol. Rev.* **59**, 1–61
- Chang, C. J., and Sonenshein, G. E. (1991) *Matrix* **11**, 242–251
- Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752
- Chirgwin, J. M., Przybyla, A., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
- Duyao, M., Kessler, D. J., Spicer, D. B., Bartholomew, C., Cleveland, J. L., Siekevitz, M., and Sonenshein, G. E. (1992) *J. Biol. Chem.* **267**, 16288–16291
- Evans, J., Moore, T., Kuehl, W. M., Bender, T., and Ting, J. (1990) *Mol. Cell. Biol.* **10**, 5747–5752
- Feinberg, A., and Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13
- Foos, G., Grimm, S., and Klempnauer, K. H. (1992) *EMBO J.* **11**, 4619–4629
- Golay, J., Capucci, A., Arsura, M., Castellano, M., Rizzo, V., and Introna, M. (1991) *Blood* **77**, 149–158
- Gonda, T. J., and Metcalf, D. (1984) *Nature* **310**, 249–251
- Gordon, D., Reidy, M. A., Benditt, E. P., and Schwartz, S. M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 4600–4604
- Greenberg, M. E., and Ziff, E. B. (1984) *Nature* **311**, 433–438
- Howe, K. M., and Watson, R. (1991) *Nucleic Acids. Res.* **19**, 3913–3919
- Hughes, A. F. W. (1942) *J. Anat.* **77**, 266–287
- Ibanez, C. E., and Lipsick, J. (1990) *Mol. Cell. Biol.* **10**, 2285–2293
- Jones, P. A., Scott-Burden, T., and Gevers, W. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 353–357
- Kelly, K., Cochran, B., Stiles, C., and Leder, P. (1983) *Cell* **35**, 603–610
- Kenyon, K., Contente, S., Trackman, P. C., Tang, J., Kagan, H. M., and Friedman, R. (1991) *Science* **253**, 802
- Kindy, M. S., and Sonenshein, G. E. (1986) *J. Biol. Chem.* **261**, 12865–12868
- Kindy, M. S., Chang, C.-J., and Sonenshein, G. E. (1988) *J. Biol. Chem.* **263**, 11426–11430
- Lam, E. W.-F., and Watson, R. (1993) *EMBO J.* **12**, 2705–2713
- Lam, E. W.-F., Robinson, C., and Watson, R. J. (1992) *Oncogene* **7**, 1885–1890
- Lawrence, R., Chang, L.-J., Siebenlist, U., Bressler, P., and Sonenshein, G. (1994a) *J. Biol. Chem.* **269**, 28913–28918
- Lawrence, R., Hartmann, D., and Sonenshein, G. (1994b) *J. Biol. Chem.* **269**, 9603–9609
- Lehrach, H., Frischauf, A. M., Hanahan, D., Wozney, J., Fuller, F., and Boedtker, H. (1979) *Biochemistry* **18**, 3146–3152
- Leof, E. B., Wharton, W., van Wyk, J., and Pledger, J. (1982) *Exp. Cell Res.* **141**, 107–115
- Liau, G., and Chan, L. M. (1989) *J. Biol. Chem.* **264**, 10315–10320
- Lichtler, A., Stover, M. L., Angilly, J., Kream, B., and Rowe, D. (1989) *J. Biol. Chem.* **264**, 3072–3077
- McConlogue, L., Gupta, M., Wu, L., and Coffino, P. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 540–544
- Miskulin, M., Dalglish, R., Kluge-Beckerman, B., Rennard, S., Tolstoshev, P., Brantly, M., and Crystal, R. (1986) *Biochemistry* **25**, 1408–1413
- Mizuguchi, G., Nakagoshi, H., Nagase, T., Nomura, N., Date, T., Ueno, Y., and Ishii, S. (1990) *J. Biol. Chem.* **265**, 9280–9284
- Moscovici, C. (1975) *Curr. Top. Microbiol. Immunol.* **71**, 79–101
- Nakagoshi, H., Kanei-Ishii, C., Sawazakai, T., Mizuguchi, G., and Ishii, S. (1992) *Oncogene* **7**, 1233–1240
- Nicolaides, N. C., Gualdi, R., Casadevall, C., Manzella, L., and Calabretta, B. (1991) *Mol. Cell. Biol.* **11**, 6166–6176
- Nomura, N., Takahashi, M., Matsui, M., Ishii, S., Date, T., Sasamoto, S., and Ishizake, R. (1988) *Nucleic Acids Res.* **16**, 11075–11083
- Poole, J. C. F., Cromwell, S. B., and Benditt, E. P. (1971) *Am. J. Pathol.* **62**, 391–413
- Reilly, C. F., Kindy, M. S., Brown, K. E., Rosenberg, R. D., and Sonenshein, G. E. (1989) *J. Biol. Chem.* **264**, 6990–6995
- Reiss, K., Traval, S., Calabretta, B., and Baserga, R. (1991) *J. Cell. Physiol.* **148**, 338–348
- Ross, R. (1993) *Science* **362**, 801–809
- Schwartz, S. M., Reidy, M., and Clowes, A. (1985) *Ann. N. Y. Acad. Sci.* **454**, 292–304
- Slack, J., Parker, M. I., Robinson, V., and Bornstein, P. (1992) *Mol. Cell. Biol.* **12**, 4714–4723
- Stepp, M. A., Kindy, M. S., Franzblau, C., and Sonenshein, G. (1986) *J. Biol. Chem.* **261**, 6542–6547
- Strauss, B. H., Chisholm, R. J., Keeley, F. W., Gottlieb, A. I., Logan, R. A., and Armstrong, P. W. (1994) *Circ. Res.* **75**, 650–658
- Takahara, K., Sato, Y., Okazawa, K., Okamoto, N., Noda, A., Yaoi, Y., and Kato, I. (1991) *J. Biol. Chem.* **266**, 13124–13129
- Tashiro, S., Takemoto, Y., Handa, H., and Ishii, S. (1995) *Oncogene* **10**, 1699–1707
- Thiele, C. J., Cohen, P. S., and Israel, M. A. (1988) *Mol. Cell. Biol.* **8**, 1677–1683
- Thompson, C. B., Challoner, P. B., Neiman, P. E., and Groudine, M. (1986) *Nature* **319**, 374–380
- Venturelli, D., Traval, S., and Calabretta, B. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 5963–5968
- Watson, R. J., Robinson, C., and Lam, E. W. (1993) *Nucleic Acids Res.* **21**, 267–272
- Wu, Y., Rich, C., Lincecum, J., Trackman, P. C., Kagan, H. M., and Foster, J. A. (1992) *J. Biol. Chem.* **267**, 24199–24206