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A-*myb* Is Expressed in Bovine Vascular Smooth Muscle Cells during the Late G_1-to-S Phase Transition and Cooperates with c-*myc* To Mediate Progression to S Phase

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The Myb family of transcription factors is defined by homology within the DNA binding domain and includes c-Myb, A-Myb, and B-Myb. The protein products of the *myb* genes all bind the Myb-binding site (MBS) [YG(A/G)C(A/C/G)GTT(G/A)]. A-*myb* has been found to display a limited pattern of expression. Here we report that bovine aortic smooth muscle cells (SMCs) express A-*myb*. Sequence analysis of isolated bovine A-*myb* cDNA clones spanning the entire coding region indicated extensive homology with the human gene, including the putative transactivation domain. Expression of A-*myb* was cell cycle dependent; levels of A-*myb* RNA increased in the late G_1-to-S phase transition following serum stimulation of serum-deprived quiescent SMC cultures and peaked in S phase. Nuclear run-on analysis revealed that an increased rate of transcription can account for most of the increase in A-*myb* RNA levels. Treatment of SMC cultures with 5,6-dichlorobenzimidazole riboside, a selective inhibitor of RNA polymerase II, indicated an approximate 4-h half-life for A-*myb* mRNA during the S phase of the cell cycle. Expression of A-*myb* by SMCs was stimulated by basic fibroblast growth factor, in a cell density-dependent fashion. Cotransfection of a human A-*myb* expression vector activated a multimerized MBS element-driven reporter construct approximately 30-fold in SMCs. The activity of c-*myb* and c-*myc* promoters, which both contain multiple MBS elements, were similarly transactivated, approximately 30- and 50-fold, respectively, upon cotransfection with human A-*myb*. Lastly, A-*myb* RNA levels could be increased by a combination of phorbol ester plus insulin-like growth factor 1. To test the role of *myb* family members in progression through the cell cycle, we comicroinjected c-*myc* and *myb* expression vectors into serum-deprived quiescent SMCs. The combination of c-*myc* and either A-*myb* or c-*myb* but not B-*myb* synergistically led to entry into S phase, whereas microinjection of any vector alone had little effect on S phase entry. Thus, these results suggest that A-*myb* is a potent transactivator in bovine SMCs and that its expression induces progression into S phase of the cell cycle.

The c-*myb* gene is the cellular progenitor of the v-*myb* oncogene, which was first identified as the transforming gene of the two independently derived retroviruses avian myeloblastosis virus and E26 (reviewed in reference 47). The c-*myb* proto-oncogene has been strongly implicated in the regulation of cell proliferation and/or differentiation of hematopoietic cells (1, 4, 13, 17, 23, 24, 25, 28, 42, 44, 48, 63, 66). Furthermore, its expression has been found in other cell lineages, including vascular smooth muscle cells (SMCs) (7, 56). The c-*myb* proto-oncogene has been demonstrated to function as a transcriptional factor in several cell systems (18, 22, 38, 49, 50, 57, 68–70). The c-Myb protein has been found to bind to the DNA consensus sequence (Myb-binding site [MBS]) [YG(A/G)C(A/C/G)GTT(G/A)] (6, 30). Based on studies with chimeric forms of c-*myb*, several functional domains of the c-Myb protein have been identified. The N terminus contains a highly evolutionarily conserved sequence that constitutes the DNA binding domain (6, 69). The homology in this region is what defines the *myb* family, as conservation is much lower in the remaining portions of the gene (53). C-terminal to the DNA binding domain is a 23-amino-acid acidic region which constitutes the transactivation domain (31, 59, 69).

Two other members of the *myb* gene family, A- and B-*myb*, have been isolated based on their products' high homology with c-Myb in the DNA binding domain (53). A-Myb shares 90% homology at the amino acid level with c-Myb in this domain. A-Myb was also found to have high homology with c-Myb in its acidic transactivation domain. Human A-*myb* mRNA is approximately 5 kb long (53) and codes for an A-Myb protein of approximately 90 kDa (27). The large size of the A-*myb* mRNA is accounted for by a long, approximately 2.5-kb 3′ untranslated region. As yet, no regulatory function, such as in determining the half-life or translational efficiency of the message, has been established for this region.

The A-Myb protein functions as a transcription factor with sequence specificity very similar or identical to that of c-Myb (21, 27). Cotransfection of A-Myb with a c-Myb-binding site-driven reporter construct has revealed that A-Myb is a strong transactivator of MBS-driven constructs (27). The *mim*-1 promoter, which is known to be regulated by c-*myb*, has also been shown to be transactivated by A-Myb in cotransfection experiments and upon stable transfection of a chicken macrophage line (21). Activity of MD-1 and lysozyme, products of other genes regulated by c-*myb*, was also induced by A-Myb. A-Myb transactivation of these reporter constructs was equal to or perhaps greater than that of v-Myb, which is itself a much more potent transactivator than c-Myb (21).

In mammals, A-*myb* has a limited pattern of expression. Murine A-*myb* was seen to be expressed at high levels only in the testes, thymocytes, B lymphocytes, and gut of the adult, with low levels present in the heart, spleen, and central nervous

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system (46, 53, 65, 67). In addition, A-*myb* expression was high in the developing nervous system and the urogenital ridge (67). In chickens, A-*myb* appears to be more ubiquitously expressed, being present in embryonic fibroblasts and a variety of hematopoietic cell lines (21). Reports on the effects of proliferation on expression of A-*myb* have been somewhat contradictory. Nomura et al. (53) showed that A-*myb* mRNA was present in several lymphocyte cell lines as well as several nonhematopoietic transformed cell lines. Golay and coworkers detected A-*myb* mRNA in resting T and B lymphocytes, and this expression was down-regulated upon mitogenic stimulation (25). The same group also determined that A-*myb* was expressed at very high levels in several Burkitt's lymphoma lines as well as in a specific subset of $CD38^+$, $CD39^-$, immunoglobulin M-negative human tonsillar B lymphocytes, which are highly proliferative (26). Recently the chicken, murine, and *Xenopus laevis* A-*myb* genes have been isolated (21, 62, 67). Studies with *Xenopus* demonstrate a high level of A-*myb* expression in the actively proliferating spermatogonial cells (62).

SMCs are the major cellular constituents of the medial layer of an artery and are responsible for maintenance of vascular tone in the adult blood vessel (reviewed in reference 58). During formation of a developing artery, SMCs produce the bulk of the matrix, which provides a structural framework for the artery. Once the artery has been fully formed, SMCs differentiate into a contractile phenotype in which they normally remain (10). In certain disease states and in response to injury, however, SMCs migrate to the intimal layer. In this environment, SMCs proliferate and produce matrix components which, in association with lipids and minerals, can result in formation of an atherosclerotic plaque capable of occluding blood flow (55, 58, 60, 64). SMCs in culture similarly dedifferentiate; they grow with a high rate of proliferation and produce significant levels of matrix components (5, 8). We have previously shown that vascular SMCs express c- and B-*myb* in the late G_1 and S phases of the cell cycle (7, 43). With SMCs in culture, antisense c-*myb* oligonucleotides inhibit entry of quiescent cells into S phase (7, 61), and heparin inhibition of cell proliferation prevents c-*myb* induction and entry into S phase (56). B-*myb* expression was shown to down-regulate matrix gene expression (43). Here we report that bovine aortic SMCs in culture express the A-*myb* gene in a cell cycle-dependent fashion; A-*myb* mRNA levels increased in the late G_1-to-S phase transition, due to an increase in the rate of gene transcription. Furthermore, coexpression of c-*myc* and A-*myb* in quiescent SMCs led to entry into S phase, suggesting that A-*myb* expression functions in SMCs as a progression factor.

MATERIALS AND METHODS

Cell culture and treatment conditions. SMC explants were derived from the aorta of female calves, as we have described previously (5). 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 U of penicillin per ml, and 100 μg of streptomycin per ml (Life Technologies, Inc.). The medium was changed every 2 to 3 days, and cells were not used beyond the fourth passage. SMC cultures were synchronized as described previously (36). Briefly, the cells were plated at low density (5×10^5 cells/150-mm^2 dish) and allowed to grow exponentially for 3 days, at which time the medium was changed to DMEM supplemented with 0.5% FBS. The 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, we have found that only 1 to 2% of SMCs deprived of serum for 72 h demonstrate significant [^{3}H]thymidine nuclear labeling (36). 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 (7, 36). Levels of histone H3.2 mRNA, an S phase-specific gene (2), and cytofluorometric measurements further confirmed cell synchrony (36). Alternatively, serum-deprived cells were stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA) in the absence or in the presence of 35 ng of insulin-like growth factor 1 (IGF-1) per ml. Where indicated, cells were treated with basic fibroblast growth factor (bFGF), which had been prepared in carrier solution (50 mM Tris, 0.3 M NaCl, 1 mM dithiothreitol, 0.05% gelatin, adjusted to pH 7.5) and filter sterilized.

RNA isolation and hybridization analysis. Total cellular RNA was isolated by the method of Chirgwin et al. (12) or with Tri-Reagent (Molecular Research Center, Inc.). Equal quantities of RNA (15 to 25 μg per lane) were denatured and separated by electrophoresis on 1.0% agarose–formaldehyde gels. Separated RNA was transferred onto a GeneScreen Plus (DuPont NEN) nylon membrane. RNA was cross-linked to the membrane by UV irradiation (Stratalinker; Stratagene) at 0.12 J/cm^2 for 30 s. For RNA stability studies, cells were treated with 30 μg of 5,6-dichlorobenzimidazole riboside (DRB) per ml, a selective inhibitor of RNA polymerase II. Probes were prepared as described previously by Feinberg and Vogelstein (19); hybridization reaction mixtures contained 1×10^6 to 2×10^6 cpm of ^{32}P-labeled DNA per ml of buffer. Unhybridized probe was removed by washing blots at 68°C with 2× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 30 min, followed by 15- to 30-min washes with 1× and 0.5× SSC, as needed. Quantitation by scanning densitometry was performed with a Molecular Dynamics 300A computing densitometer.

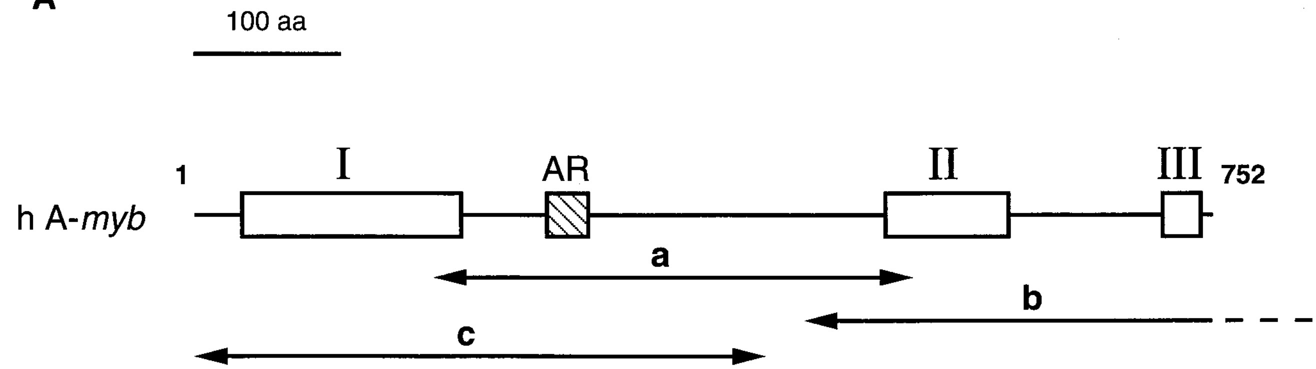
Cloning of bovine SMC A-*myb* cDNA. A reverse transcriptase (RT) reaction was carried out with 1 μg of RNA in a solution containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 μM random hexamers (Pharmacia), 1 mM (each) deoxynucleoside triphosphate (Promega), 1 U of RNase inhibitor per μl, and 2.5 U of Moloney murine leukemia virus RT per μl (Gibco BRL). Following incubation for 10 min at room temperature, samples were treated for 45 min at 42°C and then for 5 min at 95°C to inactivate the enzyme. The primer oligonucleotides were as follows: forward direction, 5′-ATGCGAAGAAAAGTGGAA CAGGAGGGCTAT-3′; reverse direction, 5′-AATGAGAGCAAAACTGCCC ACAAATAGGGGT-3′. PCR was then performed with 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM (each) deoxynucleoside triphosphate, 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus Corporation) per 100 μl, and 5 ng of each specific primer per μl, using 5 μl of the RT reaction mixture in a Thermal Cycler (Perkin-Elmer Cetus Corporation). Each cycle consisted of 90 s at 95°C followed by 60 s at 60°C and 90 s at 72°C. Normal PCRs were carried out for 30 cycles followed by a final 5-min incubation at 72°C and then incubated at 4°C until removed from the Thermal Cycler. RT-PCR fragments were cloned into the pCRII vector (Invitrogen) as described for the TA cloning kit (Invitrogen). Colonies were analyzed for inserts by restriction digestion and DNA sequencing.

A bovine A-*myb* cDNA plasmid expression vector was isolated from a custom cDNA library, constructed by Stratagene in λ-ZAPExpress with poly(A+) RNA from exponentially growing aortic SMCs and oligo(dT) and random primers. For screening, duplicate filter lifts from five plates, each containing approximately 50,000 PFU, were treated with 1.5 M NaCl–0.5 M NaOH for 2 min to denature absorbed DNA. Following neutralization for 5 min in 1.5 M NaCl–0.5 M Tris (pH 8), the filters were rinsed for 30 s in 0.2 M Tris (pH 7.6)–2× SSC and air dried, and DNA was UV cross-linked with a Stratalinker. Prehybridization was done for 4 to 16 h at 42°C, and hybridization was performed overnight in the same mixture with the inclusion of a radioactive probe. Washing was done in 2× SSC–0.1% SDS twice for 30 min each at 68°C and twice in 1× SSC–0.1% SDS for 30 min each at 65°C. Plasmid inserts were released according to the manufacturer's instructions and subjected to partial DNA sequencing to confirm identity.

DNA constructs. Histone H3.2 (pRAH3.2, a cloned genomic fragment encoding amino acids 57 to 125 of histone H3.2 [2]) and ornithine decarboxylase (ODC) (murine cDNA clone pOD48 [45]) were used. The reporter plasmid KHK-CAT-dAX was derived by insertion of nine copies of the MBS directly in front of the thymidine kinase (TK) promoter linked to the chloramphenicol acetyltransferase (CAT) gene in dAX-TK-CAT (31). The vector dAX-TK-CAT was in turn constructed from pBLCAT2 by deletion of the *Aat*II polylinker (*Xho*) fragment from the pUC18 plasmid backbone, which appeared to confer a low level of *myb*-induced transcription activity apparently caused by cryptic MBS elements (31). The vector p1.6 Bgl-CAT contains bp −1114 to +513 of the murine c-*myc* gene linked to the CAT reporter construct, as described previously (16). pHNmyb-CAT contains 1 kb of sequence upstream of the c-*myb* start site of transcription and 1.1 kb of exon 1 cloned into the pSV_0CAT reporter gene (kindly provided by T. Bender, University of Virginia School of Medicine, Charlottesville). A 3.4-kb bovine B-*myb* cDNA plasmid expression vector pB14 was isolated from the λ-ZAPExpress bovine aortic SMC cDNA library described above (43). The pKCmyb expression vector contains the *Xba*I-to-*Bgl*II cDNA fragment including the entire murine c-*myb* coding region in the pKC3/4 vector (kindly provided by R. Watson, Ludwig Institute for Cancer Research, London, England). The pCA1 vector contains the 3.1-kb *Bam*HI-to-*Hin*dIII fragment of human A-*myb* cDNA including the coding region and was subcloned into the pECE eukaryotic expression vector (27) (kindly provided by M. Introna and J. Golay, Istituto di Richerche Farmacologiche "Mario Negri," Milan, Italy).

Transfections and reporter gene assays. Cells were plated at a density of 5×10^5 cells/100-mm^2 dish 24 h before transfection. The medium was changed 2 to 4 h before transfection. DNA (50 μg) was transfected by the modified $CaPO_4$ transfection procedure of Chen and Okayama (11). The cells were harvested 48 to 72 h after transfection, and lysates were prepared as described previously (40).

A



B

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B        AGAAAGTTTTAAATCCAGAATTGATAAAGGGTCCCTGGACTAAAGAAGAAGATCAGAGGG
H   337  AGAAAGTTTTAAATCCTGAATTGATAAAGGGTCCTTGGACTAAAGAAGAAGATCAGAGGG 396

B        TTATTGAATTAGTTCAGAAATATGGGCCANAAAGATGGTCTTTAATTGCAAAACATTTAA
H   397  TTATTGAATTAGTTCAGAAATATGGGCCAAAAAGATGGTCTTTAATTGCAAAACATTTAA 456

B        AAGGAAGAATAGGCAAGCAGTGTAGAGAAAGATGGCATAATCATCTGAATCCTGAGGTAA
H   457  AAGGAAGAATAGGCAAGCAGTGTAGAGAAAGATGGCATAATCATCTGAATCCTGAGGTAA 516

B        AGAAGTCATCCTGGACAGAAGAGGAGGACAGGATTATCTATGAAGCACATAAGCGGTTGG
H   517  AGAAATCTTCCTGGACAGAAGAGGAGGACAGGATCATCTATGAAGCACATAAGCGGTTGG 576

B        GAAATCGTTGGGCAGAAATTGCCAAACTACTTCCTGGAAGGACTGATAATTCTATCAAAA
H   577  GAAATCGTTGGGCAGAAATTGCCAAACTACTTCCAGGAAGGACTGATAATTCTATCAAAA 636

B        ATCATTGGAATTCTACTATGCGGAGAAAAGTGGAGCAGGAAGCCTACTTACAAGATGGAA
H   637  ATCATTGGAATTCTACTATGCGAAGAAAAGTGGAACAGGAGGGCTATTTACAAGATGGAA 696

B        TAAAATCAGANCGATCTTCATCTAAACTTCAACACAAACCTTGTGCAACTATGGACCATT
H   697  TAAAATCAGAACGATCTTCATCTAAACTTCAACACAAACCTTGTGCAGCTATGGATCATA 756

B        TGCAAACCCAGAATCAATTTTACATACCTGTTCAGATCCCAGGCTATCAGTATGTATCAC
H   757  TGCAAACCCAGAATCAGTTTTACATACCTGTTCAGATCCCTGGGTATCAGTATGTGTCAC 816

B        CTGAAGGCAATTGTGTAGAACATGTTCAGGCTTCTTCTGCCTTTATTCAGCAACCCTTTG
H   817  CTGAAGGCAATTGTATAGAACATGTTCAGCCTACTTCTGCCTTTATTCAGCAACCCTTCA 876

B        TAGATGAAGATCCTGATAAGGAAAAAAAAATAAAGGAACTTGAGTTTCGGCTGATTTCGG
H   877  TTGATGAAGATCCTGATAAGGAAAAGAAAATAAAGGAACTTGAGATGCTTCTTATGTCAG 936

B        CTGAGAATGAAGTTAGAAGAAAACGAGTTCCATCACAACCTGGAAGCTTTTCTAGCTGGC
H   937  CTGAGAATGAAGTTAGAAGAAAGCGAATTCCATCACAGCCTGGAAGTTTTTCTAGCTGGT 996

B        CTGGTAGTTTCCTCATGGATGACAGCATGTCTAATACTCTAAATAGCCTCGAGGAGCACG
H   997  CTGGTAGTTTCCTCATGGATGATAACATGTCTAATACTCTAAATAGCCTTGACGAGCACA 1056

B        CTAGTGAGTTTTACAGTATGGATGAAAATCAGACTGTGTCTGCTCAGCAGAACTCACCTA
H  1057  CTAGTGAGTTTTACAGTATGGATGAAAATCAGCCTGTGTCTGCTCAGCAGAATTCACCCA 1116

B        CAAAGTTCCTGGCCGTGGAGGCAAACGCTGTGCTGTCCTCTCTACAGACCATCCCAGAAT
H  1117  CAAAGTTCCTGGCCGTGGAGGCAAACGCTGTGTTATCCTCTTTGCAGACCATCCCAGAAT 1176

B        TTGCAGAAACTCTCGAACTTATTGAATCTGATCCTGTAGCATGGAGTGATGTTACTAGCT
H  1177  TTGCAGAGACTCTAGAACTTATTGAATCTGATCCTGTAGCATGGAGTGACGTTACCAGTT 1236

B        TCGATCTTTCTGATGCTGCTGCTTCGCCTGTCAAGTCCACCCC
H  1237  TTGATATTTCTGATGCTGCTGCTTCTCCTATCAAATCCACCCC 1279
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FIG. 1. Characterization and analysis of the bovine A-*myb* SMC cDNA sequence. (A) A map of the complete 752-amino-acid human A-Myb protein is shown, with the alignments of three representative isolated bovine SMC A-*myb* cDNA clones (termed a, b, and c) illustrated below. The three evolutionarily conserved regions among *myb* family members are indicated as open boxes and are numbered I, II, and III. The domain rich in acidic amino acids (AR) is indicated by a hatched box. (B) Comparison of the bovine (B) and human (H) A-*myb* cDNA sequences. The sequence of the bovine A-*myb* clone c, spanning nucleotides 337 to 1279, is shown aligned to the corresponding sequence of the human A-*myb* gene. The homology between nucleotides is indicated by vertical lines. The acidic domain is indicated by an open box.

Protein concentrations of the lysates were determined with the Bradford assay as directed by the manufacturer (Bio-Rad). Equal amounts of total protein were incubated with 2.5 μCi of [^{3}H]acetyl coenzyme A (200 mCi/mmol; New England Nuclear), 50 μM acetyl coenzyme A, and 1.6 mM chloramphenicol for 4 to 8 h, and the acetylated forms were extracted with ethyl acetate and assayed by liquid scintillation counting (40).

Transcription analysis. Nuclei were isolated from SMCs, and run-on analysis was performed by a modification of the method of Greenberg and Ziff (29). Briefly, approximately 10^7 nuclei were incubated in the presence of 250 μCi of [^{32}P]UTP (3,200 Ci/mmol) for 30 min at 30°C. Labeled RNA was isolated, and equal amounts of radiolabeled RNA (4.5×10^6 cpm/ml of hybridization buffer) were hybridized to plasmid DNA (10 μg/sample) and immobilized onto GeneScreen Plus by slot blotting, followed by UV irradiation; after hybridization, the blots were washed as previously described (29).

SMC microinjection. SMCs were maintained in DMEM supplemented with 0.5% FBS for 48 h to render them quiescent. Immediately before microinjection, the medium was supplemented with 20 mM HEPES, pH 7.3, to maintain the pH when exposed to open air. Plasmids for microinjection were adjusted to 1 μg/μl in 130 mM KCl–10 mM sodium phosphate (pH 7.3) and spun at $12{,}000 \times g$ for 10 min to eliminate particulates. Solutions were introduced into borosilicate glass capillaries (0.2-μm tip diameter) with Eppendorf microloader tips. All cell nuclei in a defined grid (approximately 4 mm^2) were then microinjected with a Narishige micromanipulator under conditions of constant flow under a nitrogen pressure of 1.4 lb/in^2 at a rate of approximately 6 to 10 cells per min. Successful microinjection was estimated to occur more than 90% of the time. Following microinjection, the culture was washed with sterile phosphate-buffered saline 10 times to minimize potential contamination during microinjection and then returned to the incubator in normal medium containing [^{3}H]thymidine. After 20 h, the cells were fixed and processed for autoradiography (36).

Nucleotide sequence accession number. The entire coding region of the bovine A-*myb* cDNA has been obtained and submitted to GenBank (accession number U86617).

RESULTS

Isolation and characterization of a bovine SMC A-*myb* cDNA clone. To determine whether A-*myb* mRNA is expressed in bovine vascular SMCs, total RNA from exponentially growing aortic SMCs was amplified by RT-PCR with oligonucleotide primers from the highly conserved regions I and II of the human gene (53). A band of the expected size of 1 kb was amplified and found to specifically hybridize to a radiolabeled human A-*myb* probe in a Southern blot (data not shown). The bovine cDNA band was subcloned in the pTA cloning vector, and the resulting clone, termed clone a (Fig. 1A), was subjected to DNA sequencing. The sequence displays a high homology to the human A-*myb* gene (approximately 90%) and spans the expected regions I and II (Fig. 1A and data not shown). Clone a was then used to screen a bovine aortic SMC cDNA library to isolate larger A-*myb* cDNA clones. Screening of 250,000 λ-ZAPExpress PFU in duplicate, through three rounds of hybridization, yielded eight putative A-*myb* cDNA clones. The 5′ and 3′ ends of these clones were subjected to DNA sequencing (Fig. 1A). Clone b, spanning nucleotides 1375 to approximately 4900 relative to the sequence for the human gene (53), represents the 3′ 3.5 kb of the bovine A-*myb* gene. Clone c, spanning nucleotides 54 to 1279, represents the 5′ 1.3 kb of the A-*myb* gene and includes the AUG start codon. Thus, these two clones account for all but approximately 100 bases between nucleotides 1280 and 1375 in the coding region of the human A-*myb* gene, which are present in clone a. The sequence for the entire coding region was obtained from these clones (Fig. 1B and data not shown). Interestingly, approximately 90% homology with the human A-*myb* cDNA was noted in the region spanning nucleotides 879 to 947 (Fig. 1B) encoding an acidic domain, previously demonstrated to be necessary for transcriptional activation by the human A-*myb* gene (65). This 23-amino-acid sequence shares 87 and 92% identity with the human and *Xenopus* A-Myb proteins (53, 62), respectively (data not shown).

A-*myb* RNA expression in bovine aortic SMCs. To monitor the nature of A-*myb* RNA expression, cultures of SMCs were synchronized with the serum deprivation-stimulation protocol previously described (36) (see Materials and Methods). RNA was isolated from exponentially growing cells, as well as from serum-deprived cells in quiescence and at the indicated time points after serum stimulation of quiescent SMCs. S phase entry begins approximately 12 h after serum addition, and DNA synthesis peaks between 16 and 20 h. As seen in Fig. 2A, Northern blot analysis detected an approximately 5-kb A-*myb* mRNA in exponentially growing cells, in good agreement with the expected size (25, 53). In quiescent cells, however, the level of this mRNA was greatly decreased. A-*myb* RNA levels began to display a slight increase by 4 to 6 h following serum stimulation and remained at this level to the 12-h time point (Fig. 2A and B, and data not shown). Levels of A-*myb* RNA increased very significantly by 16 to 18 h (Fig. 2A and B), such that they were elevated five- to sevenfold by the end of S phase at 24 h after serum stimulation compared to cells in quiescence, as determined by scanning densitometry of two independent experiments. Entry into S phase was verified by the appearance of histone H3.2 RNA, an S phase-expressed gene (data not shown). Previously, we demonstrated that aortic SMCs express high levels of B-*myb* RNA in a cell cycle-dependent fashion (43). Thus, the time courses of induction of these two *myb* family RNAs were compared directly (Fig. 2B). Expression of B-*myb* RNA increased by 12 h, consistent with previous findings (43), preceding the increase in A-*myb* RNA levels observed by the 16- to 18-h time points (Fig. 2A and B). Thus, A-*myb* is expressed in SMCs in a cell cycle-dependent manner, with low levels in quiescence and early G_1 and mRNA levels increasing during the late G_1-to-S phase transition of the cell cycle.

Transcriptional regulation of A-*myb* mRNA levels. In order to determine whether changes in the rate of gene transcription could account for the observed cell cycle-dependent increase in A-*myb* mRNA levels, nuclear run-on analysis was performed (Fig. 3). Nuclei were isolated from SMCs at the 2- and 12-h time points after serum stimulation of quiescent SMCs, when cells are in the G_0-to-G_1 transition and immediately preceding the increase in RNA levels, respectively. Radiolabeled transcripts were prepared and used in run-on hybridization analysis.

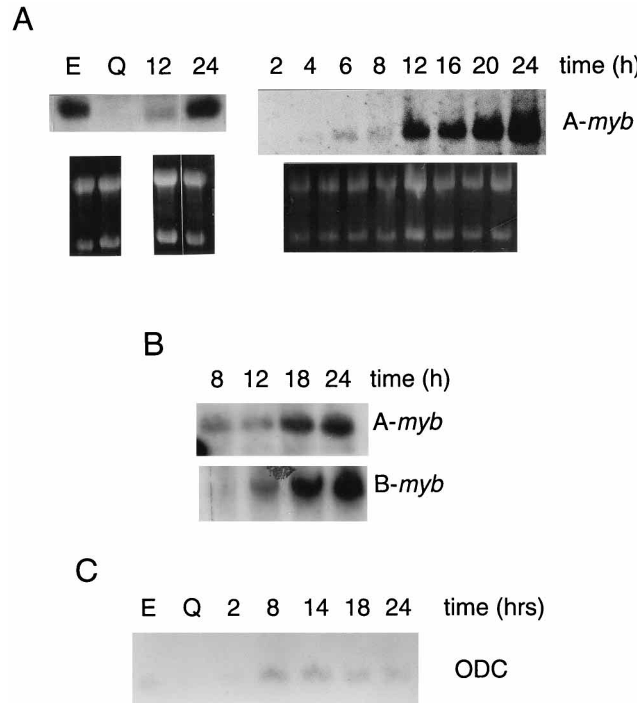

FIG. 2. Cell cycle expression of A-*myb* and ODC RNA in bovine aortic SMCs. Bovine aortic SMCs were synchronized with a serum deprivation-stimulation protocol (see Materials and Methods). Total cellular RNA was isolated from cells in exponential growth (E), in quiescence (Q), and at the indicated times (in hours) following serum addition, and samples (15 μg) were subjected to Northern blot analysis. (A) Bovine A-*myb* cDNA clone b was used as a probe with RNA from two individual experiments. Ethidium bromide staining was routinely used to confirm RNA quality and equal loading. Overloading of the 12-h sample in the right panel was noted. (B) RNA was isolated from synchronized SMC cultures and analyzed for A-*myb* and B-*myb* expression as for panel A, using bovine cDNA clones b and pB14, respectively, as probes. Equal loading was verified by ethidium bromide staining (data not shown). (C) RNA was isolated from synchronized SMC cultures and analyzed for ODC expression as for panel A. Equal loading was verified by ethidium bromide staining (data not shown).

A low level of hybridization was detected with RNA prepared from nuclei isolated 2 h after serum stimulation. A significant increase in A-*myb* gene transcription was detected 12 h after serum stimulation; this increase was four- to sixfold as judged by densitometric measurements of signal levels from this and a duplicate experiment. Only a slight induction of the rate of B-*myb* transcription was noted, consistent with the ~1.6- to 2.0-fold increase noted previously (43). As an additional control, the ODC gene was analyzed. When the RNAs prepared as described above were analyzed for ODC expression, levels of ODC mRNA began to increase at 2 h and were significantly elevated by 8 h (Fig. 2C). This finding is consistent with work of other laboratories which has demonstrated that this gene is cell cycle regulated, with increased expression occurring in the early G_1 phase (45). Thus, the increase in ODC mRNA levels precedes the rise in A-*myb* expression. Consistent with this observation, nuclear run-on analysis indicated that ODC hybridization was approximately equivalent at the 2- and 12-h time points (Fig. 3). These findings suggest that the increase in transcription of the ODC gene noted previously to occur between 0.5 and 12 h (43) occurs by 2 h following serum stimulation. Finally, pUC19 was used to verify equal RNA loading and low background hybridization to the probe backbones. Thus, the increase in A-*myb* gene transcription

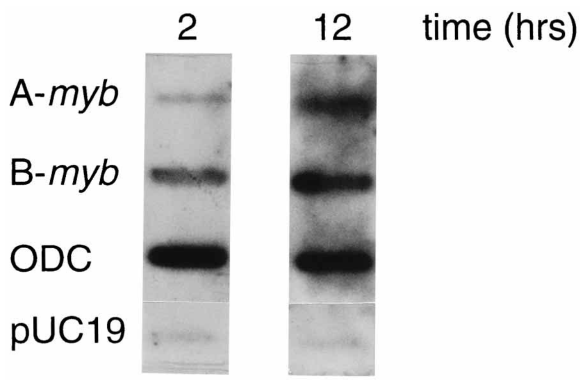

FIG. 3. Nuclear run-on analysis of cell cycle changes in the rate of transcription of the A-*myb* gene. Nuclei were isolated from SMC cultures, synchronized with a serum deprivation-stimulation protocol, 2 and 12 h after FBS restimulation and subjected to run-on analysis. Resulting radiolabeled transcripts were isolated and hybridized to 10 μg of DNA probes for the following genes, immobilized on filters: bovine A-*myb* (clone b), bovine B-*myb* (pB14), ODC, and pUC19 plasmid DNA as a control for background hybridization.

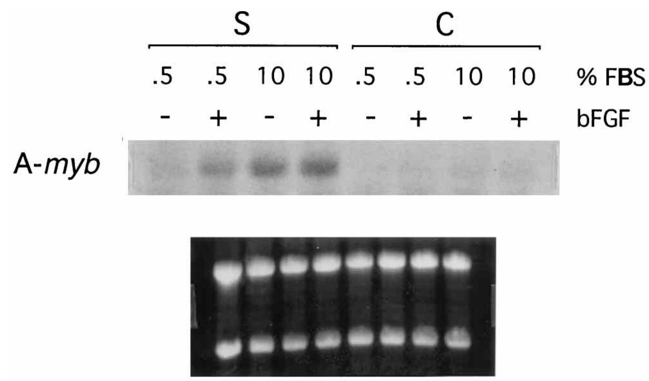

FIG. 5. Effects of bFGF on A-*myb* RNA levels as a function of cell density. SMC cultures, seeded at a density of 10^6 cells/P150 dish, were allowed to grow for either 1 or 6 days, to subconfluence (S) or confluence (C), respectively. Cells were then either maintained in complete medium, DMEM plus 10% FBS (lanes 10), or switched to serum deprivation medium, DMEM plus 0.5% FBS (lanes .5), containing either 2 ng of bFGF per ml (+) or carrier solution alone (−). RNA was isolated, and samples (15 μg) were subjected to ethidium bromide staining (lower panel) and Northern blot analysis for expression of A-*myb*.

between 2 and 12 h after serum stimulation can account for most, if not all, of the increase in the A-*myb* steady-state RNA level detected by Northern blot analysis.

A-*myb* mRNA stability in SMCs. In order to measure the half-life of the A-*myb* RNA in SMCs, cells were treated with 30 μg of DRB, a selective inhibitor of RNA polymerase II, per ml 24 h after serum stimulation during the S phase. RNA was then isolated after the indicated times of incubation with DRB, and A-*myb* and histone H3.2 mRNA levels were monitored by Northern hybridization (inset, Fig. 4). Decay in A-*myb* mRNA levels was first observed following treatment for 4 h. RNA levels were quantitated by densitometry (Fig. 4) and a half-life of approximately 4 h was calculated, similar to the value obtained for histone mRNA. Thus A-*myb* mRNA is a relatively stable message in comparison to mRNA of other transcription factors, such as c-Myc and c-Fos.

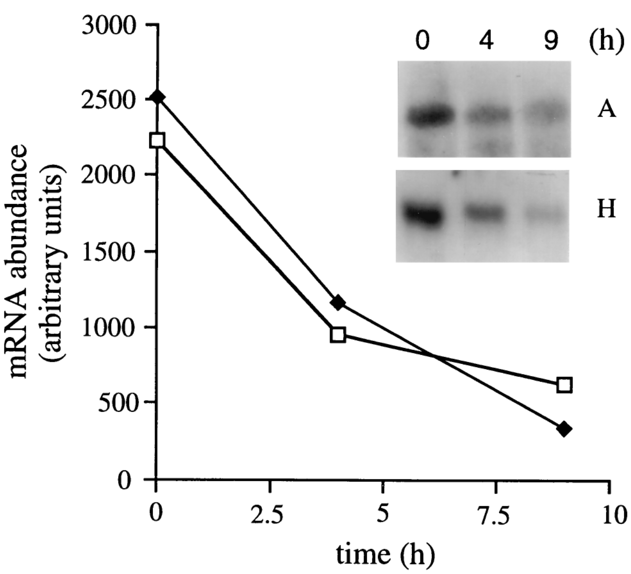

FIG. 4. Stability of A-*myb* RNA. SMC cultures were synchronized with the serum deprivation-stimulation protocol. At 24 h after serum addition, 30 μg of DRB per ml was added, and total RNA was isolated after 0, 4, and 9 h. Northern blot analysis was performed with bovine A-*myb* cDNA clone b (A) and histone H3.2 DNA (H) as probes (inset). Ethidium bromide staining confirmed RNA integrity and equal loading (data not shown). The blot was subjected to quantitation by densitometric scanning, and the results (in arbitrary units) were plotted versus time. Open squares, A-*myb* mRNA; filled diamonds, histone mRNA.

A-*myb* expression is activated by bFGF in a cell density-dependent fashion. It has been noted that bFGF is a potent mitogen for SMCs in culture (reviewed in reference 51) (data not shown); bFGF is believed to mediate stimulation of SMC proliferation in the vessel wall following injury or balloon angioplasty (37, 41). To assess the effects of bFGF on A-*myb* expression, subconfluent and confluent SMC cultures, incubated under serum deprivation conditions (DMEM plus 0.5% FBS) or in complete medium (DMEM plus 10% FBS), were monitored by Northern blotting (Fig. 5). Incubation of subconfluent cultures under serum deprivation conditions for 24 h resulted in a significant drop in the level of A-*myb* expression, suggesting that the decreased level seen in Fig. 2 occurred before the 72-h time point. Treatment of these serum-deprived subconfluent cultures with bFGF induced expression of A-*myb* RNA to almost the levels seen in normal proliferating cells (i.e., incubated in DMEM plus 10% FBS). A slight further increase in A-*myb* expression was seen upon treatment of these proliferating cells with bFGF. In contrast, growth to confluence led to a significant drop in A-*myb* mRNA levels (Fig. 5), and bFGF had little effect on the expression of this gene under conditions where growth of these cells has slowed significantly. Thus, bFGF induces A-*myb* expression in SMCs in subconfluent cultures where it promotes proliferation.

A-Myb is a potent transactivator in SMCs. Since the bovine SMC A-*myb* constructs that had been isolated were missing critical sequences necessary to address the question of functionality of A-*myb* in SMCs, a full-length human A-Myb cDNA, recently reported (27), was employed in cotransfection experiments. The homology between the products of human and bovine A-*myb* genes in the sequenced regions, including the putative activation domain, was high (approximately 90%). A construct containing nine MBSs upstream of the minimal TK promoter driving the CAT gene was used as the reporter. Cotransfection of the human A-*myb* expression vector resulted in very potent (over 30-fold) transactivation of this reporter construct in SMCs (Fig. 6A). This finding is in good agreement with results on gene activation obtained from other laborato-

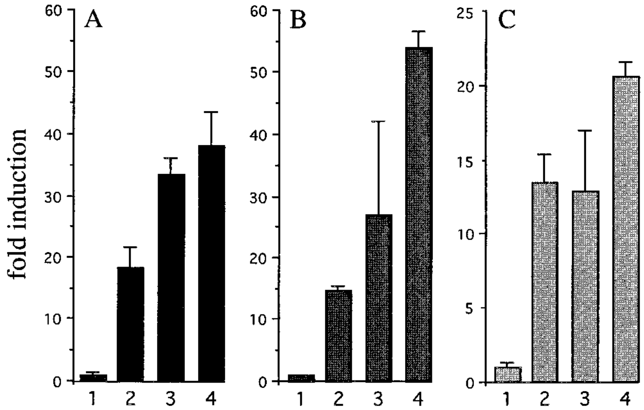

FIG. 6. Activity of A-*myb* as a transcriptional regulator in SMCs. SMC cultures were transfected in duplicate by the calcium phosphate procedure with 25 μg of the indicated CAT reporter plasmid, increasing amounts of the human A-*myb* expression vector pCA1, and enough pUC19 DNA to make up a total of 50 μg/P100 dish. Extracts containing equal amounts of protein were assayed for CAT activity. Data from one representative experiment of three experiments are shown, with values given as fold induction. Standard deviations were obtained by Student's *t* test. Lane 1, 0 μg; lane 2, 2.5 μg; lane 3, 5 μg; lane 4, 10 μg of pCA1 DNA. (A) KHK-CAT, containing nine MBS elements driving the TK promoter; (B) p1.6 Bgl-CAT c-*myc* promoter construct; (C) pHNmyb-CAT c-*myb* promoter construct.

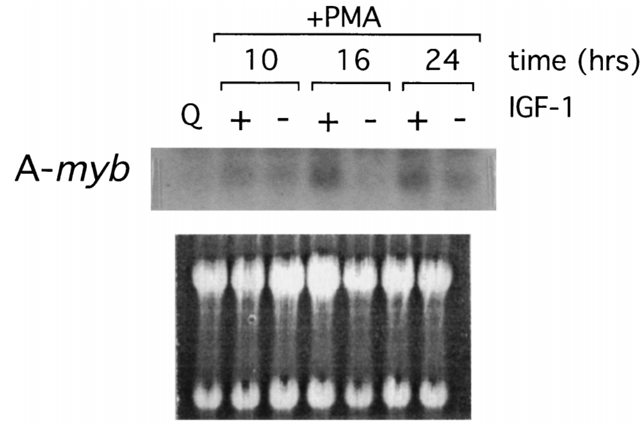

FIG. 7. Effects of PMA in combination with IGF-1 treatment on expression of A-*myb* RNA. SMC cultures were rendered quiescent by serum deprivation (DMEM plus 0.5% FBS) for 72 h. Cells were treated with 100 nM PMA in the absence (−) or presence (+) of 35 ng of IGF-1 per ml. RNA was isolated at the indicated time points as well as from cells in quiescence (Q) and subjected to Northern analysis for A-*myb* expression. Ethidium bromide staining of the gel (bottom panel) confirmed RNA quality and equal loading.

ries with A-*myb* using other cell systems (27, 67). Thus A-Myb functions as a strong transactivator in SMCs.

The c-*myc* promoter, which contains several MBSs, has been shown to be transactivated by c-Myb (14, 18, 49, 70). A similar cotransfection experiment was performed with the p1.6 Bgl-CAT c-*myc* promoter construct, which contains 1.6 kb of the c-*myc* promoter, upstream and exon 1 sequences driving CAT expression (16), including both the distal and proximal MBS elements mapped by Cogswell and coworkers (14). A-*myb* expression potently up-regulated the activity of a cotransfected c-*myc* promoter (Fig. 6B); a 50-fold up-regulation of c-*myc* promoter activity was noted. Transfection studies performed with the c-*myb* promoter have revealed that c-Myb is capable of regulating its own promoter (52). To test whether A-Myb could also regulate the c-*myb* promoter, cotransfection experiments were performed. Cotransfection of an A-*myb* expression vector with the c-*myb* promoter revealed that A-Myb up-regulated the c-*myb* promoter activity in a dose-dependent fashion, approximately 20-fold (Fig. 6C). Thus A-*myb* expression leads to transactivation of both the c-*myb* and c-*myc* promoters in transient assays.

A-*myb* can cooperate with c-*myc* to mediate progression into S phase. Previous studies have suggested that c-*myb* functions as a progression factor (23). In order to determine whether A-*myb* expression was consistent with a role in progression to S phase, we monitored the effects of IGF-1 on PMA-treated cells. PMA, which has been found to induce competence genes such as c-*fos* and c-*myc* (29, 34), requires a progression factor such as IGF-1 to induce significant levels of entry into S phase (54). As seen in Fig. 7, treatment of SMC cultures for 16 h with a combination of PMA and IGF-1 significantly induced A-*myb* mRNA levels. In contrast, in cultures treated with PMA alone, only a modest level of A-*myb* expression was induced, and this induction was delayed with respect to the dual treatment. This profile of expression is consistent with a role of A-*myb* expression in progression rather than competence.

We next asked whether A-*myb* could cooperate with c-*myc*, which has been found to act as a competence factor (3, 33), to promote entry into S phase. SMCs that had been serum deprived for 48 h were microinjected with c-*myc* or A-*myb* expression plasmids alone or in combination and then analyzed for progression into S phase by incorporation of tritiated thymidine. Cells were labeled for 20 h immediately after microinjection (Fig. 8). Autoradiography demonstrates that while expression of c-*myc* or A-*myb* alone exerted modest stimulatory effects on the percentage of cells traversing S phase (10.9% labeled nuclei with buffer alone versus 15.4 and 23.6%, respectively), coexpression of these plasmids exhibited a synergistic and quite potent stimulation of quiescent cells into S phase (74.2% labeled nuclei). When we compared all of the members of the *myb* gene family which have been found to be expressed in vascular SMCs (7, 43) in a separate experiment, both A-*myb* and c-*myb* cooperated with c-*myc* to promote DNA synthesis (Fig. 9). In contrast, B-*myb* failed to cooperate with c-*myc* to induce DNA synthesis. Furthermore, DNA synthesis did not appear to be simply delayed, as no increase in incorporation of tritiated thymidine was noted during a later, 18- to 26-h labeling window (data not shown). These data suggest that A-*myb* as well as c-*myb* can functionally cooperate with c-*myc* to stimulate entry into S phase.

DISCUSSION

The A-*myb* gene is expressed in bovine aortic SMCs in a cell cycle-dependent fashion; A-*myb* mRNA levels were low in quiescence and early G_1, increased during the late G_1-to-S phase transition, and peaked in S phase following stimulation with serum. The increase in A-*myb* mRNA levels observed during the cell cycle in SMCs appeared to be due predominantly to an increased rate of gene transcription. A-*myb* mRNA in SMCs decayed with an approximate half-life of 4 h following inhibition of RNA synthesis by DRB treatment. Mitogenic stimulation with either bFGF or PMA plus IGF-1 also induced A-*myb* RNA levels. A-Myb functioned as a potent transactivator in transient assays, leading to up-regulation of the c-*myc* and c-*myb* promoters of approximately 50- and 20-fold, respectively. Furthermore, A-*myb* functionally cooperated with c-*myc* to induce DNA synthesis in quiescent SMCs. This did not appear to result from rescue from apoptosis, since microinjection of the c-*myc* expression vector alone did not

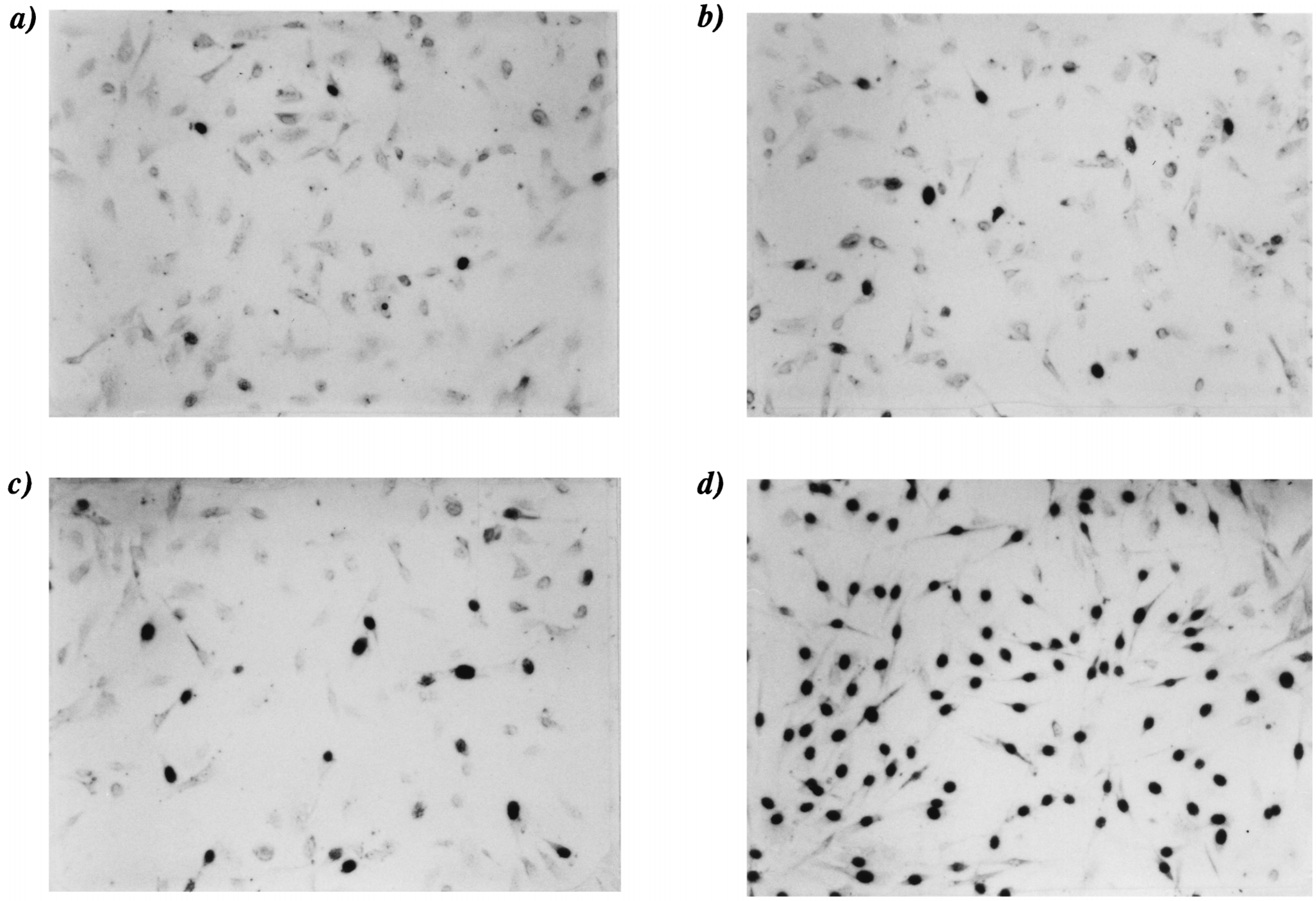

FIG. 8. Effects of A-*myb* and c-*myc* expression on entry of quiescent SMCs into S phase. SMC cultures were rendered quiescent via serum deprivation for 48 h. All cells within a field (between 157 and 244 cells per sample) were microinjected with the following vectors: none (a), 1 μg of pM21 c-*myc* expression vector alone per μl (b), 1 μg of pCA1 A-*myb* expression vector alone per μl (c), and 0.5 μg of c-*myc* expression vector per μl plus 0.5 μg of A-*myb* expression vector per μl (d). Following microinjection, the cells were washed extensively and incubated in medium containing 0.5% FBS and 2 μCi of [^{3}H]thymidine per ml. After 20 h, the cells were fixed and processed for autoradiography (36).

lead to cell death (data not shown). Although none of the bovine A-*myb* SMC cDNA clones isolated were full length and, therefore, they could not be used in the transactivation studies, A-*myb* is likely to function in a similar fashion given the very high (approximately 90%) sequence homology between bovine and human A-*myb* genes. Recently we reported that SMCs express the B-*myb* gene (43). Here, we show that induction of the levels of B-*myb* mRNA precedes that of A-*myb*. Previously, we demonstrated that pulmonary artery SMCs express c-*myb* in a cell cycle-dependent fashion, with the increase in expression detected by 8 h (7). Aortic SMCs appear to express only very low levels of c-*myb* RNA (data not shown). Thus, vascular SMCs can express all three members of the *myb* gene family during active proliferation. We have recently explored the role of B-*myb* in vascular SMCs and found that it functions as a negative transcriptional regulator. The promoters for which A-*myb* mediated a potent transactivation effect were essentially unaffected by B-*myb* (43); furthermore, B-*myb* inhibited the activity of the MBS-driven heterologous promoter construct and that of several collagen promoters (43). B-*myb* similarly functioned as a negative transcriptional regulator in 3T3 fibroblasts and hematopoietic cells (20, 68). Consistent with these findings, we observed that B-*myb*, unlike A-*myb* or c-*myb*, could not cooperate with c-*myc* to promote progression into S phase. Thus, these two genes do not appear to be functionally redundant. A question remains as to the possible redundant functions of A-*myb* and c-*myb*, which recognize and transactivate through the same binding element.

Here, we demonstrate the cell cycle-dependent expression of A-*myb* RNA in SMCs. Furthermore, with the competence-progression model worked out mainly in 3T3 fibroblasts (54), A-*myb* gene expression was found to be induced by growth factors or combinations of growth factors known to induce G_1-to-S phase progression. While A-*myb* gene expression has never been conclusively linked to the cell cycle in any other cell type, elevated A-*myb* mRNA levels have been detected selectively in proliferating cells. For example, A-*myb* RNA was detected in germinal centers, which are sites of active B-lymphocyte proliferation within the spleen, but not in the primary follicles, which contain small resting B cells (67). Furthermore, Sleeman (62) found A-*myb* expression during early stages of spermatogenesis in *X. laevis*, suggesting a function in germ cell development. Similarly, using mouse testes, Trauth and coworkers (67) detected strong A-*myb* expression in spermatogonia and spermatocytes but not in spermatids, suggesting a role for A-*myb* in proliferation and differentiation of germ cells.

The detection of A-*myb* RNA in bovine vascular SMCs is somewhat surprising, since in situ studies of A-*myb* gene expression revealed a relatively limited tissue specificity in mam-

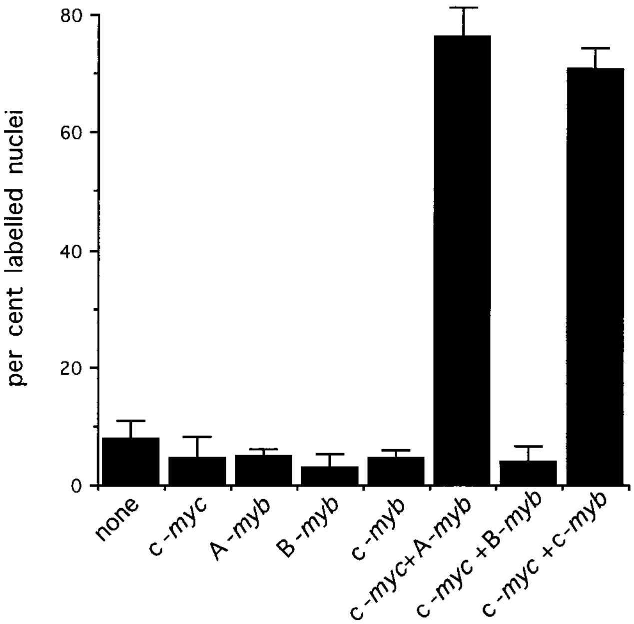

FIG. 9. Effects of *myb* family members and c-*myc* on entry of quiescent SMCs into S phase. SMC cultures, rendered quiescent as described in the legend to Fig. 8, were microinjected either with buffer alone or with the following expression vectors: pM21 c-*myc*; pCA1 A-*myb*; pB14 B-*myb*, or pKCmyb c-*myb*. Between 87 and 216 cells were microinjected per sample with 0.5 μg of expression vector DNA per μl plus 0.5 μg of Bluescript DNA per μl or with 0.5 μg of the indicated two expression vector DNAs per μl when two genes were analyzed in combination. Following microinjection, the cells were washed extensively and incubated in medium containing 0.5% FBS and 2 μCi of [^{3}H]thymidine per ml. After 20 h, the cells were fixed and processed for autoradiography. Values from one representative experiment of two experiments are given as the mean of duplicate samples ± the standard deviation.

mals (67). In the developing mouse, A-*myb* expression was predominantly detected in the developing central nervous system and the urogenital ridge; in the adult mouse, A-*myb* RNA was detected during the early stages of sperm cell differentiation and in the germinal-center B lymphocytes within the spleen. Northern blotting extended the pattern of expression, with high levels found in the testes and thymus and lower levels observed in the gut, ovaries, heart, spleen, and brain (46, 53, 67). It is likely that A-*myb* expression was not detected in blood vessel walls in the in situ studies, since they were performed at later stages of development when the SMCs in the vessel wall are likely to be in a quiescent, contractile state and would therefore not be expressing A-*myb*. Of note, we have recently observed A-*myb* expression in primary SMC cultures derived from human corpus cavernosum tissue (42a), indicating that bovine aortic SMCs are not unique or aberrant among SMC cultures in their ability to express the A-*myb* gene.

The increase in mRNA levels of A-*myb* in the cell cycle is likely due predominantly to increased gene transcription, as the four- to sixfold increase observed in nuclear run-on analysis is similar to the five- to sevenfold increase observed in Northern blot analysis. An E2F site present in the B-*myb* promoter was found to mediate cell cycle regulation of transcription of this gene (39). This is one of the first reports on A-*myb* gene transcription rates. Analysis of transcriptional control of A-*myb* awaits isolation and characterization of its promoter.

The A-Myb protein is a potent transactivator when expressed in SMCs. Transfection studies with the synthetic, MBS-driven construct KHK-CAT-dAX revealed potent transactivation by human A-Myb. This is in agreement with results obtained by other groups, which found that A-Myb is an extremely strong transactivator, much more potent than c-Myb (27, 67). Since, as discussed above, the bovine A-*myb* product exhibits high homology to the human gene product and since there have been no observed differences between species homologs among the Myb family, it is likely that bovine A-Myb is also a potent transactivator. The transactivating ability of A-Myb is equal to that of v-Myb in transfection studies (21). Other than this difference in transactivational potency, no functional distinction has been made between c-Myb and A-Myb, in terms of DNA binding, transactivation, or gene regulation. The fact that both proteins appear able to bind to the same consensus DNA binding site and transactivate the same promoters leads to the question of their functional redundancy. Unfortunately, little is known about the determinants of specificity of the Myb proteins. For example, subtle changes in the v-Myb protein expressed in hematopoietic cells changed the phenotype of the transformed cell, and Introna and coworkers showed that the three amino acid differences in the DNA binding domain between the avian myeloblastosis virus and E26 v-Myb proteins determine whether or not *mim*-1 is induced (32). The finding that A-Myb transactivates the c-*myb* promoter approximately 20-fold raises the possibility of positive regulation among the members of the *myb* family. Until the A-*myb* promoter is cloned, it will not be possible to know if there is also autoregulation of A-*myb* gene expression, although it appears that B-*myb* is not regulated in this way (39).

The c-*myc* promoter has also been shown to contain functional MBS elements (18). Cotransfection studies revealed that both c-Myb and v-Myb can transactivate the mouse and human c-*myc* promoters (14, 18, 49, 70). Gel shift and footprinting analysis with partially purified c-Myb protein revealed that c-Myb was indeed able to bind to these sites. In contrast, B-Myb was also found to be able to bind to the MBS elements in the c-*myc* promoter but was unable to transactivate it in 3T3 cells (68). We have similarly found that B-Myb failed to transactivate the c-*myc* promoter in SMCs (43), while A-Myb induced the c-*myc* promoter–CAT construct over 50-fold. The cell cycle expression patterns of A-*myb* and c-*myc* appear to preclude any role for A-*myb* in stimulation of c-*myc* gene expression in the transition from G_0 to G_1 when c-*myc* mRNA levels peak at 2 h (36) or during the early G_1 phase. It is possible, however, that induction of c-*myc* by A-*myb* may be dependent upon a posttranslational modification, e.g., phosphorylation, or other factors that are expressed in a cell cycle-specific fashion. Synergistic cooperativity in the activation of the *mim*-1 promoter between the products of the c-*myb* and c-*ets* (15) and the v-*myb* and C/EBP (9) genes has been reported. The c-Myb protein was similarly found to synergistically interact with the Epstein-Barr virus BZLF1 transactivator in lymphoid cells (35). This might explain the requirement for comicroinjection of expression vectors for both c-*myc* and A-*myb* in quiescent cells to obtain cell cycle progression. Additional experiments are required to address the intriguing possibility of a role for A-*myb* in maintenance of c-*myc* levels in the late G_1 and S phases, since in normal, cycling cells, c-*myc* levels are maintained at a constant, measurable level throughout the cell cycle (36).

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