Expression of the c-myb Proto-oncogene in Bovine Vascular Smooth Muscle Cells*

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Previously we have shown that bovine vascular smooth muscle cells (SMCs) express c-myb mRNA (Reilly, C. F., Kindy, M. S., Brown, K. E., Rosenberg, R. D., and Sonenshein, G. E. (1989) J. Biol. Chem. 264, 6990–6995). Here we have characterized changes in the low level of c-myb mRNA expressed in quiescent serum-deprived subconfluent SMCs upon entry into the cell cycle. After serum stimulation, levels of c-myb mRNA increased 3–4-fold during late G1 and remained at this level during S phase. A 1.5-kilobase partial c-myb cDNA clone, isolated from a bovine SMC library, was partially sequenced and found to be 89 and 86% homologous to the human and murine c-myb genes, respectively. Using bovine and murine c-myb clone(s) no change in the rate of c-myb gene transcription or mRNA stability was detected during the cell cycle. Thus, the regulation of changes in c-myb mRNA levels in SMCs appears distinct from mechanisms seen in hematopoietic or fibroblastic cells. Vectors containing myb binding sites linked to the thymidine kinase promoter and the chloramphenicol acetyltransferase reporter gene were transiently transfected into SMC cultures. KHK-CAT-dAX, which contains nine concatenated myb binding sites, exhibited 7-fold more activity than the parental dAX-TK-CAT vector in exponentially growing SMCs. The levels of chloramphenicol acetyltransferase activity in exponentially growing cells were approximately 2-fold higher than in cells that had been serum deprived for 24 h and were entering quiescence. Thus SMCs produce a functional c-myb protein that can activate transcription from a heterologous promoter. Furthermore, introduction of antisense c-myb oligonucleotides to quiescent serum-deprived SMC cultures severely inhibited entry of cells into S phase upon serum addition. Thus, expression of the c-myb oncogene plays an important role in cell cycle progression of 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). Subsequent demonstration of c-myb gene amplification has implicated this oncogene in human acute myelogenous leukemias (Gonsalves and Metcalf, 1984). In normal cells, high levels of c-myb mRNA are observed only in immature hematopoietic cells, and these levels decrease upon differentiation (Gonsalves and Metcalf, 1984). Furthermore, levels of c-myb mRNA have been detected in embryonal neural tissue as well as in neuroblastosomas (Thiele et al., 1988) and in chick embryo fibroblasts (Thompson et al., 1986). In earlier studies we showed that c-myb mRNA is also expressed in bovine vascular smooth muscle cells (SMCs) (Reilly et al., 1989).

Experiments indicate that the c-myb proto-oncogene plays an important role in the control of proliferation. For example, a c-myb antisense oligomer inhibited T-lymphocytes from proliferating in response to phytohemagglutinin by preferentially blocking cells in the late G1 phase of the cell cycle without blocking early cell cycle or mid-G1 events (Gewirtz et al., 1989). Cell cycle synchrony studies with hematopoietic cells have shown that c-myb expression is low in quiescent cells and increases in mid- to late-G1 and remains elevated in S phase. For example, stimulation of resting human T-lymphocytes resulted in maximal c-myb mRNA levels in mid-G1 phase and highest protein levels in S phase (Stern and Smith, 1986; Lipsick and Boyle, 1987). In chick embryo fibroblasts, Thometson et al. (1989) demonstrated that c-myb mRNA levels begin to increase approximately 4 h after serum addition. These results suggest that c-myb expression plays a role in cell cycle progression during G1 phase important for entry into S phase.

The proteins encoded by v-myb and c-myb can bind directly to specific sequences of DNA (PyACCm-m2(G)) through three highly conserved amino acid sequences in the amino-terminal portion of the myb protein (Biedenkapp et al., 1988). In the DNA binding domain of the c-myb protein, there are three tryptophans in each repeat which appear to be important for association with the DNA (Saikumar et al., 1990; Kane-Ishii et al., 1990). A region in the carboxyl terminus is responsible for activating transcription (Weston and Bishop, 1989). Both v-myb and c-myb expression vectors activate transcription when co-transfected with a reporter plasmid containing multiple copies of the binding sequence (Nishina et al., 1989; Klemppenauer et al., 1989). Although myb binding sites have been identified within the SV40 enhancer and the long terminal repeat of human immunodeficiency virus type 1, only one cellular gene to date, mim-I, has been shown to be activated by myb expression (Ness et al., 1989).

Previously we observed that treatment with heparin, which inhibited the entry of SMCs into S phase, blocked expression of c-myb mRNA.
of c-myb mRNA with little effect upon c-fos and c-myc mRNA levels (Reilly et al., 1989). Here we have characterized the expression of c-myb mRNA in cultured bovine vascular SMCs. We find that the low level of c-myb mRNA in quiescent serum-deprived SMCs increases upon serum addition during late G1, and inhibition of expression of this oncogene prevents entry into S phase; furthermore, trans-activation of a reporter plasmid containing myb binding sites indicates the functional role of this proto-oncogene in SMC transcriptional control.

MATERIALS AND METHODS

Cell Culture Conditions—Smooth muscle cell explants were obtained from the pulmonary artery of female calves as described previously by Stepp et al. (1986). Cells were passaged after 4-7 days in second passage. Second- and third-passage cells were used for experiments. Tissue culture reagents were purchased from Gibco, except for Dulbecco’s modified Eagle’s medium (DMEM), which was purchased from Hazleton. Cells were synchronized with the serum deprivation-stimulation protocol described previously (Kindy and Sonenshein, 1986). Briefly, cells were plated at an initial density of 5 × 10^5 cells/100 dish. These subconfluent cultures were incubated for 3 days in medium supplemented with 10% fetal calf serum (FCS) (10% FCS-DMEM) to permit exponential growth. They were then incubated in medium supplemented with 0.5% FCS and 10 mM HEPES (0.5% FCS-HEPES) for an additional 3 days. The cells were then stimulated by addition of fresh medium containing 15% FCS. DNA synthesis was assessed during a 2-h labeling period with 2 μCi/ml [3H]thymidine (Du Pont-New England Nuclear; 80 Ci/mmol) using autoradiography to determine the percent labeled nuclei as described (Campisi et al., 1984). For studies on the effects of sense and antisense oligonucleotides, the procedure of Holt et al. (1988) was followed. Cells were synchronized as above except that 20 μM oligodeoxynucleotide was added during the last 24 h of incubation in 0.5% FCS-DMEM and upon restimulation with fresh medium containing 15% FCS and 2 μCi/ml [3H]thymidine for a 24-h labeling period. Cells were fixed and analyzed as above for percent cells in DNA synthesis.

RNA Hybridization and Nuclear Run-off Analyses—Total RNA was isolated by the LiCl-urea procedure (Auffray and Rougeon, 1980). Equal quantities of RNA were subjected to electrophoresis on 1% agarose-formaldehyde gels, and Northern blots were hybridized and washed as described previously (Dean et al., 1988). The probes were made using random-primed cDNA inserts (Feinberg and Vogelstein, 1982). Mobilities are in kilobases and were calculated as described previously (Lehrach et al., 1977). For mRNA stability studies, 5 μg/ml actinomycin D (Boehringer Mannheim) or 20 μg/ml 5,6-dichloro-β-D-ribofuranosylbenzimidazole (Sigma) was employed. Inhibition of incorporation of [3H]uridine into RNA was measured 10 min after the addition of inhibitor to the cultures during a 1-h incubation of 2 μCi/ml [3H]uridine (ICN; 36 Ci/mmol). Trichloroacetic acid-precipitable counts were assayed by liquid scintillation counting. Nuclear run-off analysis was performed using the method of Green and Ziff (1984) as we have described previously (Kindy and Sonenshein, 1986). The DNA probes employed in these studies were myb: "4626 containing the 3′ coding region and hybridizes to all actin mRNAs (Bond and Farmer, 1983).

Expression of the c-myb Proto-oncogene in SMC Cultures—To characterize the cell cycle expression of the c-myb gene in SMCs, RNA was isolated from cells in exponential growth, quiescence, and 2 and 18 h after serum stimulation. Northern blot analysis was performed using MM49, a murine cDNA c-myb clone. Exponentially growing cells expressed significant levels of a 3.8-kilobase c-myb mRNA (Fig. 1a). In contrast, c-myb RNA was present at very low levels in quiescent cells or in cells 2 h after serum stimulation. By 18 h, the c-myb mRNA levels had increased significantly. A more complete time course indicated that c-myb mRNA levels began to increase 8

The time for serum addition is expressed as the mean of triplicate cultures rounded to the nearest integer. ND, not done.
c-myb Expression in Smooth Muscle Cells

h after serum stimulation and reached maximal levels by 16-20 h (Fig. 1b). Therefore, the increase in c-myb mRNA occurs in mid- to late G1, immediately prior to the onset of DNA synthesis; maximal expression occurs during S phase.

Isolation of a Bovine c-myb cDNA—Since cross-hybridization of the murine c-myb cDNA to the 28 S ribosomal band, which is only slightly larger than the c-myb RNA, made quantitation of c-myb mRNA difficult, a bovine c-myb cDNA clone was isolated. A cDNA library constructed with bovine SMC RNA isolated late in G1 (11 h after serum stimulation) was screened using a 32P-labeled murine c-myb clone (Nomura et al., 1988). Interestingly, although M2A does not include the DNA binding domain of c-myb, regions of homology between M2A and human (H) genes (Bender and Kuehl, 1986; Majello et al., 1988). A dot indicates every 10 bases in the sequence. Panel c, alignment of M2A with murine c-myb. Domains of murine c-myb are indicated. bp, base pairs.

A-myb and B-myb are two human cDNAs recently cloned from a T-cell and breast cancer cell line library because of their similarity to c-myb sequences in the DNA binding domain; however, their similarity in other regions is not great (Nomura et al., 1988). Interestingly, although M2A does not include the DNA binding domain of c-myb, regions of homology between M2A and A- and B-myb were noted (Fig. 3). Specifically, 76% homology was noted between M2A and A-myb over a region of 151 bases whereas 78% homology was observed over a 23-base region in c-myb and B-myb. Thus these regions of myb may have been conserved during evolution to preserve a necessary function of the protein.

Functionality of c-myb Protein—To test whether SMCs synthesized a functional myb protein, a transfection experiment was performed using the previously identified myb-DNA
binding motif with a heterologous promoter and reporter construct. The plasmid KHK-CAT-dAX was derived from dAX-TK-CAT by insertion of nine copies of the myb binding site, TAAACGG, directly in front of the thymidine kinase promoter linked to the chloramphenicol acetyltransferase gene (Ibanez and Lipsick, 1990). The dAX-TK-CAT vector was in turn constructed from pBLCAT2, by deletion of the AatII-polylinker (XhoI) fragment from the pUC18 plasmid backbone since it appears to confer a low level of myb-induced transcription activity apparently caused by cryptic sites (Ibanez and Lipsick, 1990). A negative control, pBLCAT3, a derivative of pBLCAT2 which does not contain the thymidine kinase promoter, was used. All three plasmids were transfected into exponentially growing SMCs; co-transfection with the dAX-TK-CAT control plasmid was observed, indicating c-myb protein activation of this thymidine kinase promoter in SMC cultures.

Cells were either refed with 10% FCS-DMEM to keep them growing exponentially or washed and shifted to 0.5% FCS-DMEM to make them begin to become quiescent. Cell lysates, prepared 24 h later, were analyzed for protein content and β-galactosidase activity.

With lysates from exponentially growing cells, few differences in transfection efficiencies were noted as judged by β-galactosidase activity. The percent conversion for KHK-CAT-dAX was 4.2%, that for dAX-TK-CAT was 0.6%, and pBLCAT3 was 0.3% (Fig. 4). Thus, a 7-fold increase in activity of the KHK-CAT-dAX compared with the dAX-TK-CAT control plasmid was observed, indicating c-myb protein activity of this thymidine kinase promoter in SMC cultures.

In cells that were deprived of serum, a consistent decrease in β-galactosidase activity was seen compared with those in exponential growth. Since the cells were transfected and then split, these differences could not be related to transfection efficiency. When protein concentrations were determined, almost twice the amount of protein was detected in lysates from cells in exponential growth compared with those deprived of serum. Equal amounts of protein lysate were used from a comparison of cells incubated in different serum concentrations. An approximate 1.7-fold decrease was observed between the percent chloramphenicol acetyltransferase conversion in cells transfected with KHK-CAT-dAX or dAX-TK-CAT incubated in 10% FCS-DMEM compared with those in 0.5% FCS-DMEM (Fig. 4). In three experiments, the decrease ranged from 1.7- to 2.9-fold. If one corrects for differences in protein content/cell, then lysates from exponentially growing cells display 3.4-5.8-fold higher levels of chloramphenicol acetyltransferase activity compared with those in 0.5% FCS. Thus, the myb protein activity is higher in exponentially growing cells than in cells that are becoming quiescent, consistent with changes in c-myb mRNA levels.

Expression of c-myb and Entry into S Phase—To assess whether expression of c-myb is critical for progression of SMCs through the cell cycle, the effect of antisense c-myb oligonucleotides on the entry of quiescent cells into S phase was measured. Two regions of the c-myb gene were chosen for oligonucleotide synthesis. Oligonucleotide 1 was derived from the bovine sequence within exon 3, near the exon 3/intron 3 splice junction (Table I). Oligonucleotide 2 was prepared against the start site of translation of the mouse and chicken c-myb genes. This sequence was selected since it displays 100% identity between various species (Bender and Kuehl, 1986; Gerondakis and Bishop, 1986), and it encompasses the human sequence successfully employed by Gewirtz and Calabretta (1988) in their studies of hematopoietic cells. The addition of 15% FCS-DMEM to quiescent serum-deprived control cells stimulates approximately 95% of the cells to undergo DNA synthesis as judged by nuclear labeling during

![Figure 4](image_url)

**Fig. 4.** Chloramphenicol acetyltransferase activity of myb reporter plasmids. Chloramphenicol acetyltransferase activity of SMC cultures after transfaction with either KHK-CAT-dAX (KHK-CAT), parental dAX-TK-CAT, or control pBLCAT3, normalized for DNA uptake using co-transfection of pMSV-β-gal. Lysates were prepared from cells in exponential growth (10%) or after serum deprivation for 24 h (0.5%).

**Table II**

| Effect of sense and antisense oligonucleotides on SMC proliferation | DNA synthesis numbers are reported as mean ± S.E. of quintuplicate cultures. Sense 1: 5′-ACATTTCTGAAGCACAC-3'; antisense 1: 5′-GTTTGTGCTTCAGAATGT-3'; sense 2: 5′-ATG GCCCGGAGACCCC-3'; antisense 2: 5′-GGGTCTCGGGGCAT-3′. | Oligonucleotide | % Cells in DNA synthesis |
|---|---|---|
| Study 1 | Control 1 (no serum) | 2.84 ± 0.38 | |
| | Control 2 (serum, no oligomer) | 94.48 ± 1.56 | |
| | c-myb sense 1 | 87.30 ± 2.24 | |
| | c-myb antisense 1 | 31.92 ± 0.79 | |
| | c-myb sense 2 | 88.80 ± 2.39 | |
| | c-myb antisense 2 | 28.52 ± 1.39 | |
| Study 2 | Control 1 | 3.68 ± 0.98 | |
| | Control 2 | 94.54 ± 1.79 | |
| | c-myb sense 1 | 88.92 ± 1.34 | |
| | c-myb antisense 1 | 28.56 ± 0.92 | |
| | c-myb sense 2 | 92.08 ± 1.16 | |
| | c-myb antisense 2 | 29.15 ± 0.91 | |
| Study 3 | Control 1 | 3.14 ± 0.53 | |
| | Control 2 | 95.32 ± 1.13 | |
| | c-myb sense 1 | 90.80 ± 1.83 | |
| | c-myb antisense 1 | 43.56 ± 2.28 | |
| | c-myb sense 2 | 88.52 ± 2.17 | |
| | c-myb antisense 2 | 29.32 ± 1.52 | |
a 24-h labeling period (Table II), consistent with results presented above. In contrast, the presence of either antisense oligonucleotide severely inhibited entry into S phase, only approximately 30% of the cells replicated their DNA. As a control, the corresponding sense oligonucleotides were compared; they had very little effect on the number of cells undergoing DNA synthesis (Table II). Thus, the addition of specific oligonucleotides to inhibit c-myb gene function to cultures of quiescent SMCs prevents their progression through S phase.

Regulation of c-myb mRNA Levels—To evaluate the transcriptional component of the changes in c-myb mRNA, nuclei were isolated from cells during exponential growth, quiescence, and 2, 4, and 18 h after serum stimulation. Gene transcription was examined by nuclear run-off assays (Fig. 5). A low level of hybridization to the c-myb probe was observed with no significant change in the rate of transcription detected over the time course. Similar results were obtained using the M2A c-myb CDNA insert and with additional time points of 8, 12, and 18 h (data not shown). Since both MM46 and M2A contain only sequences beyond the first exon, downstream of the mapped site of elongational control, hybridization should be a measure of mRNA synthesis (Bender et al., 1987). In contrast, transcription of histone H3.2 and actin genes varied as expected based on our previous results (Kindy and Sonenshein, 1986). Transcription was low in quiescent cells compared with exponentially growing ones and increased by 18 h when the cells were in S phase. The failure to observe changes in transcription of the c-myb gene suggests that the changes observed in c-myb mRNA expression are not caused by transcriptional activation.

To determine whether an increase in RNA half-life could account for elevated c-myb mRNA expression during the late G1 phase, the stability of c-myb mRNA in exponentially growing SMCs and during cell cycle progression was assayed. During exponential growth, quiescence, and 8 and 20 h after serum addition, cells were treated with the inhibitor of transcription actinomycin D (5 μg/ml) for lengths of time up to 3 h. The efficacy of the inhibitor was assayed by [3H]uridine incorporation and was found to inhibit more than 95% incorporation into trichloroacetic acid-precipitable cpm. In exponentially growing cells, no decay of the c-myb mRNA was observed even after 3 h of actinomycin D treatment (Fig. 6). As the SMCs moved from quiescence to S phase, the c-myb mRNA remained very stable throughout the time course (Fig. 6). In contrast, the level of histone H3.2 mRNA clearly decayed during the 3-h treatment, consistent with known lability of this mRNA. Similar results were obtained using the selective inhibitor of RNA polymerase II, dichlorobenzimidazole (data not shown). Therefore, the c-myb mRNA was relatively stable in SMC cultures; furthermore, changes in stability probably do not account for the observed increases in RNA expression of this gene during G1 to S phase transition.

DISCUSSION

Bovine vascular smooth muscle cells express the c-myb oncogene. In quiescent serum-deprived cells, c-myb mRNA levels are low; after serum addition, the levels increase as cells enter late G1 phase and peak in S phase. The expression of a functional protein, as measured by chloramphenicol acetyltransferase assays, roughly parallels that of the mRNA, being higher in exponentially growing cells than in cells that are becoming quiescent. Furthermore, introduction of c-myb antisense oligonucleotides severely inhibits entry of SMCs into S phase. These results suggest that c-myb expression is required for cell cycle progression of the SMC to DNA synthesis.

The expression of c-myb mRNA in SMCs is unusual since c-myb mRNA so far has been shown to be expressed only in cells of hematopoietic lineage and in embryonic and transformed cells. The level of c-myb mRNA expression is low in SMCs compared with hematopoietic cells; however, its expression was confirmed by the isolation of a CDNA clone from a bovine SMC library. Furthermore, the murine myb probe hybridized to an mRNA of the correct size for c-myb and did not cross-hybridize with A-myb or B-myb mRNAs, whose sizes are 5.0 and 2.6 kilobases, respectively (Nomura et al., 1988). Similar to what has been reported in a number of other cell types, c-myb mRNA increases during late G1 and peaks during S phase of the cell cycle in SMC. In normal human T-lymphocytes, when Gewirtz et al. (1989) blocked c-myb RNA using a similar antisense oligonucleotide strategy, they observed that cells traversed the G1 phase and were specifically blocked from entry into S phase. Interestingly, inhibition of SMC proliferation by heparin, which blocks the cells in late G1 in vivo (Castellot et al., 1985), blocks the expression of c-myb RNA but not the early increase in c-fos or c-myb mRNA in vitro (Reilly et al., 1989). Travali et al. (1991) have shown recently that constitutive c-myb expression in Balb/c 3T3 cells induced insulin-like growth factor-1 mRNA production, thereby abating the need for exogenous insulin-like growth factor-1 for cell growth. Therefore, expression of c-myb protein appears to play a role in helping cells progress from G1 phase through to S phase.

The c-myb protein binds DNA and can trans-activate transcription of heterologous promoters-reporter gene constructs containing myb binding sites (Ibanez and Lipsick, 1990; Klemplauer et al., 1989). Given that the positive sequence is only 6 base pairs long, it is likely that surrounding sequences will play a role in control of trans-activation. The myb protein may also act as trans-repressor by binding to an alternate DNA sequence (Nakagoshi et al., 1989). At this time, however, only the mim-1 gene, which encodes a promyelocytic cell secretary protein, has been identified as having myb binding
sites within its promoter (Ness et al., 1989). Since expression of mim-1 is cell type specific, additional factors may be involved in mediation of c-myb trans-activation. Furthermore, c-myb DNA binding activity can be regulated by protein phosphorylation, suggesting another level of control of trans-activation function (Luscher and Eisenman, 1990). Transfection of KHK-CAT-dAX indicates that c-myb protein in SMC can activate transcription in a DNA binding-dependent fashion. The role of c-myb during the cell cycle remains to be determined.

In most hematopoietic cells, c-myb RNA is regulated by control of the rate of elongation of gene transcripts (Bender et al., 1987). A site has been mapped in the first intron where the RNA polymerase seems to pause, similar to what happens in the c-myc gene (Bentley and Groudine, 1986). In neuroblastoma cells, c-myb mRNA abundance also seems to be regulated by transcription (Thiele et al., 1988). However, c-myb RNA levels in chick embryo fibroblasts are controlled by message stability (Thompson et al., 1986). When c-myb mRNA is increasing in abundance, the RNA is more stable than when its levels are decreasing (246 min compared with 28 min). In SMC neither the level of gene transcription nor the stability of the mRNA appeared to change upon cell cycle progression. The c-myb mRNA appeared quite stable in SMCs compared to the rapid decay of this message seen normally in fibroblasts and hematopoietic cells; furthermore, no change in stability was detected during the cell cycle. The possibility of the RNA products should help determine if this novel mechanism of regulation of c-myb gene expression occurs in the SMC.

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