The intracellular concentration of ionized calcium is involved in regulating mitosis. However, little is known about intracellular levels of calcium during G1. We have demonstrated in vascular smooth muscle cells a mid-G1 decrease in ionized calcium concentration followed by a 2-fold rise at the G1/S interface (44 nM ± 0.6 nM versus 98 nM ± 1.1 nM, p < 0.01). The elevation of intracellular calcium is preceded by an increase in c-myb mRNA levels and is abolished with antisense but not missense c-myb oligonucleotides. Furthermore, cells stably transfected with c-myb show a similar 2-fold augmentation in intracellular calcium concentrations, as compared with untransfected cells, which is also abolished by antisense c-myb oligonucleotides. The c-myb-induced rise in intracellular calcium is dependent upon the presence of extracellular calcium and is not suppressed by L type calcium channel blockers. We conclude that c-myb induces an elevation in intracellular calcium levels of vascular smooth muscle cells at the G1/S interface which provides a novel role for this proto-oncogene as well as a potentially important control point for cell cycle regulation.

The intracellular levels of calcium are involved in controlling cell cycle progression and cell growth. It is widely appreciated that transient increases of intracellular calcium occur early in mitosis and during anaphase (1, 2). These elevations appear to be required for disappearance of the nuclear envelope, condensation of chromosomes, breakdown of mitotic spindles, and activation of the contractile ring (3-6). The regulation by intracellular calcium concentrations of the G1 to S transition is less thoroughly documented. However, earlier investigations have suggested that the divalent cation may be required for entry of cells into S phase (7, 8). With these latter results in mind, we measured the intracellular ionized calcium concentrations of vascular smooth muscle cells (SMC) 1 early in the cell cycle and then ascertained whether the proto-oncogene c-myb which is differentially expressed at this time might be responsible for the observed variations.

The proto-oncogene c-myb is homologous to the transforming gene product of the avian myeloblastosis virus and was originally thought to be present only in hematopoietic cells (9, 10). However, c-myb and its various homologues are synthesized by diverse cell types (11, 12). The expression of this proto-oncogene occurs at low levels in quiescent cells, increases rapidly as cells begin to proliferate, and peaks in the late G1 phase of the cell cycle (13, 14). In some biologic systems, c-myb plays an important role in regulating cellular differentiation and growth. Thus, introduction of exogenous vectors expressing high levels of the proto-oncogene block differentiation of myeloid cells (15) whereas treatment with antisense c-myb decreases growth of human myeloid leukemic cell lines in vitro (16). The proto-oncogene is also intimately involved in controlling the proliferation of vascular SMC. Thus, antisense c-myb oligonucleotides suppress proliferation of vascular SMC in vitro (17, 18) as well as in vivo, 2 with the block occurring in late G1. In the present study we show that the proto-oncogene is critically involved in regulating intracellular calcium concentrations of vascular SMC in a cell cycle-specific fashion and speculate that the observed effect may be important for cell growth.

MATERIALS AND METHODS

Stable Transfections—The mouse c-myb cDNA LTR-driven eukaryotic expression vector pS090 was kindly provided by Dr. M. Kuehl (NCI, NIH), and the human c-myb cDNA SV40-myc expression vector PMBm1 was a gift from Dr. E. Prochorowik (University of Michigan, Ann Arbor, MI). SV40LT-SMC, an immortalized rat vascular smooth muscle cell line, was obtained from Dr. C. Reilly (Merck, Sharpe and Dohme, West Point, PA) (19). The mouse LTA cell line was used for control transfections (20). Neomycin resistance genes were removed from the two c-myb expression constructs, and modified plasmids were then transfected into SV40LT-SMC and LTA cell lines with a hygromycin resistance vector pGMV/HyTK (21) at a 15:1 molar ratio using the calcium phosphate precipitation technique (22). After overnight incubation with calcium phosphate precipitate, the cells were washed twice with PBS, trypsin treated, and plated at clonal density in Belco 24-well dishes (Belco, Vineland, NJ). Selection with hygromycin, 200 µg/ml, was initiated 24 h later and continued until the emergence of colonies. The colonies were allowed to increase in size to approximately 1 mm in diameter and then were trypsin treated and plated in 75-mm² tissue culture dishes (Belco). The various clones were subsequently analyzed by Southern and Northern hybridization for the presence of genomic and RNA myb sequences. A representative clone from each group (LTR-MYB and SV40-MYB) was extensively employed for further studies, whereas three additional myb-transfected clones (two SV40-driven and one LTR-driven) were utilized less frequently.

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The abbreviations used are: SMC, smooth muscle cells; LTR, long terminal repeat; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.
**RNA Analysis**—For Northern blot analysis, total RNA was prepared from transfected and untransfected cells by the method of Chomczynski and Sacchi (23). Samples of 15 µg of RNA/lane were run on 1% formaldehyde-agarose gels and transferred to GeneScreen Plus membranes (Du Pont) according to the manufacturer's recommendations. UV cross-linking (UV Stratalinker 1800, Stratagene Cloning Systems, La Jolla, CA) and hybridized with a random-primer c-myb cDNA probe (specific activity of 1–2 × 10^6 cpm/µg) at 42 °C for 24 h in a Gene Screen Plus hybridization solution prepared to the manufacturer's specifications. The blot was then washed twice in 2× SSC, 0.1% SDS at 24 °C, and 2× SSC, 0.1% SDS at 55 °C and then analyzed quantitatively with a Betascope 605 Blot Analyzer (Betagen, Waltham, MA).

For dot blot analysis, samples of 20 µg of total RNA were applied to Gene Screen Plus membranes prestreaked in 20 × SSC, UV cross-linked, and then hybridized with a random primed c-myb cDNA probe (specific activity of 1–2 × 10^6 cpm/µg). The washing and hybridization conditions were the same as described previously. The amounts of applied RNA were normalized by hybridization of blots with a GAPDH cDNA probe (specific activity of 1–2 × 10^6 cpm/µg) and then analyzed as outlined above.

**Quantitative Immunoprecipitation**—The c-myb-transfected as well as untransfected SV40LT-SMC cells were plated in 100-mm tissue culture dishes and allowed to become confluent in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal bovine serum. The cells were washed three times with PBS, plated for 2 h in methionine-free DMEM supplemented with 10% fetal bovine serum, 1% Triton X-100, 50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM MgCl2, 150 mM NaCl, 10 mM phenylmethylsulfonyl fluoride, 100 mM iodoacetate, and incubated for 20 min on ice. The samples were then spun at 15,000 g for 10 min at 4 °C, lo6 counts (in vitro synthesized c-myb) in the same bands.

Determinations of Intracellular Calcium with Fura-2 and Indo-1—The cells were plated at a low density on glass cover slips (for Fura-2 imaging) or cluster-6 tissue culture dishes (for Indo-1 flow cytometry) and allowed to attach overnight. The dishes were then washed twice with saline and changed to DMEM supplemented with 0.5% fetal bovine serum. The cells were analyzed for Fura-2 or Indo-1 fluorescence after 96 h of starvation (growth arrest) and at various time points after the shift to DMEM supplemented with 10% fetal bovine serum. In antiserum experiments, 25 µM antisense c-myb phosphorothioate oligonucleotides (GTGTCCGGGGTCTCCGGGC, nucleotides 4–22 of the mouse sequence) were added at the time of serum stimulation as described previously (17). Corresponding 18-base pair mismatched controls (GTGCCGGGCTTCTCGGGC) phosphorothioate oligonucleotides were also used as controls.

The Fura-2 and Indo-1 acetylomethyl (AM) esters (Molecular Probes, Eugene, OR) were dissolved in anhydrous dimethyl sulfoxide and stored at −20 °C in light-tight tubes. For cell loading, 10 µl of 1 mM Fura-2 AM or 1 mM Indo-1 AM was added to 2 ml of Hanks' solution supplemented with 1 mM CaCl2 and 1.1 mM MgCl2. The loading buffer also contained 0.01% Pluronic F127 (BASF Wyandotte Corporation, Wyandotte, MI). Incubations were carried out for 30 min at 34 °C; cells were washed twice with Hanks' buffer and allowed to de-esterify dyes for at least 30 min at 24 °C in a light-tight container. For determinations in the calcium-free media, cells were processed as described above except that media were changed 5 min before examination to 1 mM calcium containing Krebs buffer calcium-free Krebs buffer, or PBS supplemented with 2 mM EGTA.

Intracellular calcium determinations were performed as described previously (17). Incubations were carried out for 30 min at 34 °C; cells were washed twice with Hanks' buffer and allowed to de-esterify dyes for at least 30 min at 24 °C in a light-tight container. For determinations in the calcium-free media, cells were processed as described above except that media were changed 5 min before examination to 1 mM calcium containing Krebs buffer calcium-free Krebs buffer, or PBS supplemented with 2 mM EGTA.

**RESULTS**

**DNA Flow Cytometry**—DNA flow cytometric analyses were carried out with SMC loaded either with propidium iodine or Hoesch 3342 (Molecular Probes). Prior to staining with propidium iodine, cells were washed with PBS, trypsin treated, and resuspended for 10 min at 25 °C in 500 µl of hypotonic lysis solution (0.1% Triton X-100, 0.1% sodium citrate, 100 µg/ml propidium iodine). The cells were spun for 5 min at 2,000 X g, resuspended for 5 min at 37 °C in the same solution with 2 mg/ml RNase A, 30 µl of 5 M NaCl was added, and suspensions were passed through a 100-µm nylon filter. Flow cytometry was conducted on a Coulter EPICS C flow cytometer (Coulter Systems, Hialeah, FL). Loading with Hoechst 3342 was carried out by adding 4 µl of 10 µM solution of the dye to 4 ml of cell culture and incubating for 1 h at 37 °C. The cells were then washed, trypsin treated, and resuspended in 2 ml of DMEM containing 5 mM dye. Flow cytometry was performed on a FACStar Flow Cytometer. The raw cell cycle data for both propidium iodine- and Hoesch 3342-stained cells were analyzed using a MODFIT program (Verity Software, Topsham, ME; release 5.0).

**RESULTS**

We employed a large T-immortalized rat aortic SMC line, SV40LT-SMC, to investigate changes in intracellular calcium levels during cell cycle progression. To this end, SMC were grown in serum deprivation for 96 h and then stimulated by the addition of 10% fetal bovine serum. The concentrations of intracellular ionized calcium were determined at 8-h intervals using the fluorescent indicators, Fura-

3H
2 and Indo-1. The data obtained by full-field image analysis of representative cells using Fura-2 demonstrate that intracellular calcium levels are unchanged for the first 8 h, decline at 16 h, rise to the initial levels at 24 h, and drop back again at 32 h (Fig. 1, top). The results generated by flow cytometry using Indo-1 confirm the time-dependent alterations in intracellular calcium concentrations as well as the homogeneity of the response in the entire cell population. Flow cytometric analysis of cellular DNA shows partial cell cycle synchronisation and reveals that increased intracellular calcium levels at 24 h occur as the cell population enters S phase (Fig. 1, bottom). Identical experiments using Indo-1 have been carried out with primary rat aortic SMC isolated as described previously (12) with similar changes in intracellular levels of ionized calcium.

The concentrations of c-myb mRNA in SV40LT-SMC were determined by dot blot analysis with normalization to glyceraldehyde phosphate dehydrogenase mRNA which demonstrate that message levels are low in growth-arrested cells (0 h), increase significantly at 16 h (late G1), and reach a maximum at 24 h (G1/S interface) (Fig. 2). Thus, the increased concentrations of proto-oncogene mRNA precede the elevation in intracellular calcium levels (as determined with Fura-2). The measurements of intracellular calcium in SV40LT-SMC were repeated after addition of antisense or missense c-myb oligonucleotides prior to serum stimulation. The concentrations of antisense oligonucleotide selected had previously been shown to inhibit proto-oncogene expression and block S phase progression in this cell culture system (17). These experiments demonstrate that addition of antisense c-myb oligonucleotide, as compared with missense c-myb oligonucleotide, almost completely suppresses elevated [Ca2+]i, levels seen at 24 h (Fig. 2) but not at 0 h (see Fig. 5). Therefore, the increased concentrations of intracellular calcium at G1/S interface appear to require the presence of increased levels of the proto-oncogene.

We attempted to establish a direct relationship between the expression of c-myb and increased levels of intracellular calcium. To this end, we stably cotransfected SV40LT-SMC and mouse LTA cells with full-length LTR-driven mouse c-myb (p3090) or SV40-driven human c-myb (pMBM1) constructs in conjunction with the antibiotic resistance vector pUCM/HyTK. Cells stably expressing either set of constructs were selected by growth in hygromycin, and individual clones were examined for either p3090 or pMBM1 by Southern blotting with the entire c-myb cDNA. Digestion with BamHI demonstrated that the various clones exhibit different sites of integration, no gross rearrangements had occurred, and copy numbers average one or two per haploid genome (data not shown).

The concentrations of c-myb mRNA were determined in two representative clones, p3090-4 (LTR-MYB) as well as pMBM-1 (SV40-MYB) and in untransfected control cells. Total cellular RNA was isolated from growth-arrested cells, exponentially growing cells, as well as those predominantly at the G1/S interface. Northern analyses were carried out using a random primed 2.4-kilobase BamHI-HindIII restriction fragment from plasmid p3090 which detects transfected human and mouse as well as endogenous rat sequences (Fig. 3A). The equivalent loadings of samples were documented by ethidium bromide staining and hybridization with glyceraldehyde phosphate dehydrogenase cDNA. The gel electrophoretic patterns were quantitated with a Betagen 603 blot analyzer. Based upon two independent experiments, exponentially growing LTR-MYB and SV40-MYB express levels of c-myb mRNA, normalized to glyceraldehyde phosphate dehydrogenase mRNA, which are 3.0- and 5.5-fold greater than

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**Fig. 1.** Intracellular calcium levels during cell cycle progression in vascular smooth muscle cells. **Top,** the concentrations of intracellular calcium in SV40LT-SMC determined with Fura-2 following serum stimulation (mean ± S.E.). **Bottom,** cell cycle distribution determined in parallel samples.

**Fig. 2.** Effect of antisense c-myb oligonucleotide on elevated calcium levels at the G1/S interface. The concentrations of intracellular calcium (light bars) and the levels of c-myb mRNA (dark bars) in SV40LT-SMC at 0, 16, and 24 h after serum stimulation. The missense or antisense phosphorothioate c-myb oligonucleotides were added to cultures at a concentration of 25 μM at the time of serum stimulation (24-h time point). The data are displayed as mean ± S.E.
untransfected cells predominantly at the G1/S interface. Furthermore, the concentrations of c-myb mRNA were equivalently high in transfected clones when measured at growth arrest and 24 h after serum stimulation.

The rates of synthesis of c-Myb protein were determined in exponentially growing LTR-MYB and SV40-MYB as well as SV40LT-SMC predominantly at the G1/S interface. To this end, the various cell lines were labeled for 1 h with [3H]leucine and [35S]-labeled c-Myb protein immunocaptured from untransfected SV40LT-SMC (third lane) and 35S-labeled c-Myb protein immunocaptured from untransfected SV40LT-SMC in the presence of 10 µg of peptide against which the c-myb antibody was raised (second lane), and 35S-labeled c-Myb protein immunocaptured from untransfected SV40LT-SMC in the absence of added peptide (third lane).

We analyzed the levels of intracellular ionized calcium in c-myb-transfected as well as untransfected control cells under different growth conditions. Full-field imaging with Fura-2 shows that growth-arrested transfected cells, as compared with growth-arrested untransfected cells, exhibit an elevated but relatively homogeneous distribution of calcium concentrations (Fig. 4). This observation was confirmed by Indo-1 flow cytometry in a qualitative manner. The measurements of intracellular calcium concentration in c-myb-transfected clones (LTR-MYB and SV40-MYB) show a substantial increase in Fura-2 ratios in growth-arrested as well as predominantly late G1 clones as compared with untransfected control cells at the same stage of the cell cycle (Fig. 5). Using in vivo and in vitro calibration techniques, the observed elevations of Fura-2 ratios in growth-arrested LTR-MYB and SV40-MYB, as compared with growth-arrested untransfected control cells, well as electrophoretically separating proto-oncogene proteins as outlined above, and determining the levels of 35S/[3H]-labeled 79- and 92-kDa c-Myb protein as outlined under "Materials and Methods." Based upon two independent experiments, exponentially growing LTR-MYB and SV40-MYB exhibit rates of synthesis of c-Myb protein which are 1.9- and 3.6-fold greater that untransfected cells predominantly at the G1/S interface. The inclusion of 35S counts within the 45-kDa band would not change the above conclusion. These results are in reasonable agreement with the levels of c-myb mRNA in the various cell types. A preliminary examination of three additional clones (one LTR-driven and two SV40-driven) yielded virtually identical data.

The levels of proto-oncogene protein were quantitated by adding known amounts of in vitro translated [3H]leucine-labeled c-myb to the 35S-labeled c-myb, immunocapturing as
correspond to an average 1.8-fold increase in the levels of intracellular calcium (158 ± 15.6 nM versus 92 ± 9.6 nM, p < 0.05). The same results as described above were generated with three additional c-myb-transfected clones (Fura-2 ratios for LTR-MYB and SV40-MYB clones average 1.37 ± 0.16 as compared with similarly designated ratios for untransfected cells of 1.02 ± 0.01). Identical alterations in the intracellular concentrations of calcium were also noted in growth-arrested stably transfected LTA cells as compared with untransfected LTA cells (69.7 ± 9.9 nM versus 32.0 ± 1.7 nM, p < 0.01).

We also exposed growth-arrested LTR-MYB and untransfected cells to 25 μM antisense c-myb oligonucleotide or antisense 2-base pair mismatch c-myb oligonucleotide. The treatment of growth-arrested LTR-MYB with antisense c-myb oligonucleotide, but not 2-base pair mismatch oligonucleotide, completely abolishes elevated concentrations of intracellular calcium, whereas the treatment of similarly designated untransfected control cells with antisense c-myb oligonucleotide had no effect on the levels of intracellular calcium (Fig. 5). Northern analysis showed a 2.5-fold reduction in the concentrations of c-myb message in growth-arrested LTR-MYB after exposure to antisense c-myb oligonucleotide, as well as the lack of effect on proto-oncogene message with antisense 2-base pair mismatch c-myb oligonucleotide.

We then attempted to determine whether elevated concentrations of intracellular calcium induced by c-myb are secondary to altered influx from extracellular sources or altered efflux from intracellular compartments. To resolve this issue, we measured the levels of [Ca2+]i in growth-arrested LTR-MYB and similarly designated untransfected control cells placed for 5 min in 1 mM calcium-containing Hanks' buffer, calcium-free Krebs buffer, and PBS supplemented with 2 mM EGTA. The latter treatment would be expected not only to prevent influx of extracellular calcium, but also to empty stores of the cation in the sarcoplasmic reticulum. The growth-arrested LTR-MYB and untransfected control cells exhibited a decline in the concentrations of intracellular ionized calcium to the same final levels (Fig. 6). The exposure of growth-arrested LTR-MYB to 1 or 3 μM dihydropyridine nifedipine (added in the dark), a specific blocker of L type calcium channels, had no effect upon proto-oncogene-dependent elevations of intracellular calcium (Fura-2 ratios to nifedipine-free controls: 0.98 ± 0.1 (no nifedipine); 0.94 ± 0.1 (1 μM nifedipine); 1.02 ± 0.15 (3 μM nifedipine), p = not significant).

**DISCUSSION**

We measured the concentrations of intracellular calcium in SMC partially synchronized with respect to the cell cycle. The results show that intracellular calcium levels decrease as cells transit from G0 to G1, increase for a prolonged period of time to the starting concentrations as cells enter S phase, and undergo a subsequent prolonged drop to levels typical of G1 at later points in the cell cycle. The elevated concentrations of intracellular calcium at G0 are observed in the absence of significant levels of c-myb, and the addition of antisense or sense proto-oncogene oligonucleotides does not affect intracellular calcium levels. The increased concentrations of intracellular calcium at the G1/S interface occur in conjunction with augmented synthesis of c-myb, and the addition of antisense or sense proto-oncogene oligonucleotides, but not sense proto-oncogene oligonucleotides, specifically suppresses the above alterations of divalent cation. The latter result is observed when c-myb concentrations at the G1/S interface are reduced by about 70% to levels below that found in late G0. Thus, elevations of intracellular calcium concentrations at the G1/S interface appear to be under the control of c-myb, whereas the molecular events responsible for increased concentrations of intracellular calcium at G0 and decreased intracellular calcium levels at G1 are unknown. However, c-myb might function in a very indirect manner to raise intracellular calcium levels by allowing cells to progress to a point in the cell cycle which is associated with elevations of divalent cation.

To exclude this possibility, we determined whether expression of c-myb could directly elevate intracellular calcium concentrations independent of growth state. This was accomplished by stably transfecting SV40LT-SMC with the proto-
oncogene, demonstrating that increased levels of c-myb mRNA as well as protein were produced, and then measuring the concentrations of intracellular calcium. The growth-arrested c-myb-transfected clones, as compared with growth-arrested untransfected control cells, exhibit increasing levels of intracellular calcium which is virtually identical in magnitude to that noted at the G1/S interface. Similar results were obtained when c-myb-transfected cells in late G1 were compared with untransfected cells at the same stage of the cell cycle. We eliminated potential experimental artifacts by employing different promoters to drive expression of c-myb and by utilizing transfected clones with different insertion sites. In addition, we used antisense proto-oncogene oligonucleotides to suppress the increased levels of intracellular calcium in the transfected clones to those of the untransfected cells, which indicates that our observations were not secondary to biased selection of transfected clones. Furthermore, we also stably expressed c-myb in LTA cells which produced elevated intracellular calcium concentrations and excluded the possibility that interactions of the transfected proto-oncogene with large T antigen present in immortalized vascular SMC were responsible for our observations. It is of interest to note that the concentrations of c-myb in growth-arrested transfected cells and in untransfected cells predominantly at the G1/S interface differ by 2- to 4-fold, whereas the intracellular calcium levels are virtually identically elevated. Furthermore, higher levels of transfected proto-oncogene do not correlate with greater elevations of intracellular calcium. These observations suggest that concentrations of c-Myb protein must exceed a threshold which is set at about two times late G1 levels of proto-oncogene to raise intracellular calcium, but additional elevations of c-Myb protein exert minimal effect on intracellular divalent cation.

We then demonstrated that the c-myb-dependent elevations of intracellular calcium are likely to be generated by increased influx of the cation from the external environment rather than increased efflux of the cation from an internal compartment. However, the increased entry of calcium does not appear to occur via L-type channels because nifedipine is unable to suppress the observed alterations and is unlikely to take place via T-type channels since these structures are not usually present in vascular SMC. The augmented calcium influx could be due to a direct effect of c-myb on a novel calcium channel or an unknown calcium transporter. It is also possible that the proto-oncogene could exert an indirect effect on well characterized calcium exchangers or antiporters. For example, c-myb might decrease function of the Na/K ATPase which would increase entry of calcium via the Na/Ca exchanger or decrease function of the Na/H exchanger which could augment influx of calcium via a Ca/H antiporter. The structure of c-Myb is similar to a "typical" DNA-binding protein with an amino-terminal helix-loop-helix domain as well as a carboxyl-terminal leucine zipper motif (9). The proto-oncogene binds to specific DNA sequences and regulates transcription of known genes such as c-myc and mim-1 (28, 29). Therefore, c-Myb is likely to increase concentrations of intracellular calcium by a transcriptional mechanism which alters synthesis of a component of a cation transport system or changes production of a protein that modifies activity of a cation transport system.

We believe that elevated concentrations of intracellular calcium at the G1/S interface produced by increased expression of c-myb may be essential for cell cycle progression. Prior investigations have demonstrated that calcium deprivation of many eukaryotic cells, including yeast, induces a temporary growth arrest in late G1 (7, 8, 30). Previous studies have also revealed that transiently increased levels of calmodulin accelerate various cell types through the G1/S interface, whereas transiently decreased calmodulin levels slow progression of the above cell types through the same point in the cell cycle (31, 32). In preliminary experiments, we have reversed the G1 arrest of SMC induced with antisense c-myb oligonucleotide by adding small amounts of the calcium ionophore 4-bromo-A23187 16 h after serum stimulation. Thus, the normal transition of cells from G1 to S phase appears to be dependent upon threshold levels of intracellular calcium and/or calmodulin. Based upon the available evidence, we suggest that the proto-oncogene elevates intracellular calcium concentrations at late G1 which regulates S phase progression of SMC as well as other cell types. This novel mechanism would provide a specific growth regulatory function for c-myb and might also explain the effects of the proto-oncogene on cell differentiation.

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REFERENCES
1. Keith, C. H., Ratan, R., Maxfield, F. R., Bajer, A., and Shelanski, Z. (1986) Nature 324, 464-465.
2. Poenie, M., Alderton, J., Steinhardt, R., and Tsien, R. (1986) Science 232, 896-899.
3. Steinhardt, R. A. and Alderton, J. (1988) Nature 332, 364-366.
4. Twigg, J., Patel, R., and Whitaker, M. (1989) Nature 332, 366-369.
5. Sissen, J. E., Silver, R. B., Barrows, G. H., and Graesch, D. (1985) in Advances in Microscopy (Cowden, R. R., and Harrison, F. W., eds) pp. 73-87, Alan R. Liss, New York.
6. McIntosh, J. R., and Koonce, M. P. (1989) Science 246, 622-628.
7. Paul, D., and Risten, H. J. (1979) J. Cell. Physiol. 96, 31-40.
8. Pardee, A. B., Duhrow, R., Hamlin, J. L., and Kletten, R. F. (1978) Annu. Rev. Biochem. 47, 715-730.
9. Luscher, B., and Eisenmann, R. N. (1990) Genes & Dev. 4, 2235-2241.
10. Anfossi, G., Gewirtz, A. M., and Calabretta, F. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5379-5383.
11. Thompson, C. B., Challoner, P. B., Neiman, P. E., and Groudine, M. (1986) Nature 319, 374-380.
12. Relly, C. F., Kindey, M. S., Brown, K. E., Rosenberg, R. D., and Sonenshein, G. G. (1988) J. Biol. Chem. 263, 6990-6995.
13. Stern, J. B., and Smith, R. (1986) Science 233, 202-206.
14. Lipsija, J. S., and Boyle, W. J. (1987) Mol. Cell. Biol. 7, 3358-3360.
15. Clarke, M. F., Kukowska-Latallo, J. F., Westin, E., Smith, M., and Pro-2767, 656-662.
16. Gewirtz, A. M., and Calabretta, F. (1988) Proc. Nat. Acad. Sci. U. S. A. 87, 9784-9788.
17. Lupton, S. (1991) Mol. Cell. Biol. 11, 3374-3378.
18. Sambrook, J., Pittac, F. S., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
20. Papageorgiou, J., and Morgan, K. G. (1991) Am. J. Physiol. 260, H507-H515.
21. Moore, R. D. W., Becker, P. L., Fogarty, K. E., Williams, D. A., and Fay, P. S. (1990) Cell Calcium 11, 157-179.
22. Gordon, G. J., Lipsija, J. S., and Baluda, W. A., (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 685-689.
23. Smith, I. L., Diri, P. W., Lipsija, J. S. (1991) 7th Annual Meeting on Onogene: Foundation for Advanced Cancer Studies, p. 3, Bethesda, MD.
24. Smol, J. L., Moors, T. L., Kushi, W. M., Bender, T., and Ting, J. P. (1990) Mol. Cell. Biol. 10, 5747-5752.
25. Dube, K. T., Tantravahi, R. R., Rao, V. N., Reedy, E. S., and Reddy, F. P. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1291-1295.
26. Takaokas, J. G., Bolton, W. E., Hidaka, H., Boyd, A. E. J., and Means, A. R. (1992) Cell 69, 1-11.
27. Rasmussen, C. D., and Means, A. R. (1989) EMBO J. 8, 73-82.