c-Myb-binding Sites Mediate G1/S-associated Repression of the Plasma Membrane Ca^{2+}-ATPase-1 Promoter*

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Talat Afrozé and Mansoor Husain:
From the Centre for Cardiovascular Research, 3-816, 101 College Street, Toronto General Hospital, Toronto, Ontario M5G 1L5, Canada

We demonstrate that two Myb-binding sites of the mouse plasma membrane Ca^{2+}-ATPase-1 (PMCA1) promoter are required for G1/S cell cycle stage-associated repression of PMCA1 promoter activity. Nuclear run-on experiments revealed G1/S-associated repression of PMCA1 transcription. Ribonuclease protection assays revealed two transcription initiation sites between two point mutated Myb binding sequences of the PMCA1 promoter. Transient transfection assays using cell cycle-synchronized vascular smooth muscle cells (VSMC) and PMCA1 promoter-luciferase constructs showed a 2-fold decrease in reporter activity at G1/S as compared with G0. Overexpression of wild-type c-Myb severely repressed PMCA1 promoter activity at both G0 and G1/S while co-transfection of a dominant negative c-Myb, or a construct encoding an anti-c-Myb neutralizing antibody, completely abolished the repression seen at G1/S. Single nucleotide substitutions in the first, second, or both Myb-binding sites alleviated the G1/S-associated transcriptional repression of the PMCA1 Ca^{2+} pump in rodent VSMC by direct binding to the PMCA1 promoter.

The myb family of transcription factors is a polyphyletic group whose members possess a conserved DNA-binding domain with a helix-turn-helix like motif (recently termed the “Myb box”) (1). Myb box proteins carry out a variety of functions including positive and negative transcriptional regulation, modulation of mRNA stability (2), and the regulation of telomere length (3). They have diversified enormously in plants where they represent the largest known regulatory gene family (4). In vertebrates, only three family members (A-, B-, and c-myb) are known, of which c-myb and its encoded gene product c-Myb have been well characterized. The c-Myb DNA-binding domain recognizes the consensus hexanucleotide sequence (C/T)AAC(G/T)G. c-Myb also contains transactivation and C-terminal negative regulatory domains. c-Myb expression is vitally important for the control of cell proliferation in a variety of cell types, and c-Myb is also involved in the regulation of apoptosis (reviewed in Ref. 5). Vascular smooth muscle cells (VSMC) express c-Myb at the late G1 phase of the cell cycle (6, 7). VSMC are dynamic structural and functional components of blood vessel walls. As contractile cells they are capable of effecting vascular tone. However, as proliferative cells they participate in vasculo-occlusive processes such as atherosclerosis and post-angioplasty restenosis (8). In studying the mechanisms regulating VSMC proliferation, we have focused on the role played by c-Myb in the regulation of intracellular Ca^{2+} concentrations ([Ca^{2+}]_i) at the G1/S cell cycle interface. We have shown that c-Myb activity regulates [Ca^{2+}]_i, in both VSMC and fibroblasts (9–11). Experiments employing either wild-type or dominant negative forms of c-Myb, in which cell cycle-associated Ca^{2+} homeostasis was monitored, implicated the plasma membrane Ca^{2+}-ATPase (PMCA) family of Ca^{2+} efflux pumps as critical mediators of c-Myb-dependent [Ca^{2+}]_i (9, 10).

The PMCA family is high affinity, low capacity, Ca^{2+} pumps that extrude cytosolic Ca^{2+} to the extracellular space (12–14). Each of the 4 known PMCA genes (PMCA1–4) gives rise to multiple isoforms through alternative splicing (14, 15). These genes differ in their 5’ untranslated regions (14, 16) which may contain regulatory sequences that direct tissue-specific expression. PMCA1 and PMCA4 are the ubiquitously expressed members of this 4-gene family (17, 18) and Western blot analysis has shown PMCA1 expression to exceed that of PMCA4 (19).

It is known that Ca^{2+} transporting membrane proteins play a major role in modulating Ca^{2+} signaling (20). Indeed, the resting [Ca^{2+}]_i in many cell types is critically regulated by the level of PMCA activity (9, 21, 22). However, despite the obvious importance of PMCA to cellular Ca^{2+} handling, little is known of the mechanisms regulating their expression. A study on rat endothelial cells suggested that PMCA1 is regulated at the transcriptional level via a protein kinase C-dependent pathway (23). Our studies on VSMC showed that expression levels of PMCA1 are inversely correlated with levels of c-Myb activity, such that suppression of c-Myb activity led to 2–4-fold increase in the level of PMCA1 mRNA and protein (10). These manipulations resulted in a 30% reduction in mean resting [Ca^{2+}]_i, a 42% decrease in mean S phase entry, and a 36% decrease in mean cell proliferation rate of synchronized rat VSMC populations (10). We also demonstrated that a direct 2-fold overexpression of PMCA1, independent of any manipulation of c-Myb activity, resulted in similarly significant reduc-

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† Recipient of a Clinician Scientist Award from the Medical Research Council of Canada. To whom correspondence should be addressed: EN-12-221, 200 Elizabeth St., Toronto General Hospital, Toronto, ON M5G 2C4, Canada. Tel.: 416-340-3188; Fax: 416-340-4021; E-mail: mansoor.husain@utoronto.ca.
tions in \([\text{Ca}^{2+}]_{i}\), \(G_{i}\) to S transitions, and rate of cell proliferation (9). Having demonstrated the physiological importance of \(PMCA1\) regulation in VSMC, we now examine more closely the molecular interaction between c-Myb and the \(PMCA1\) gene.

Du et al. (24) have previously cloned and sequenced 1010 bp of the mouse \(PMCA1\) promoter. In their series of primer-extension and promoter-reporter assays carried out in mouse neural cells, they found the \(PMCA1\) promoter to lack a TATA box, possess numerous SP1 sites, one long GC repeat within a CpG island, and an untranslated first exon that was at least 19 kilobases away from the translational start within exon 2. We have re-cloned and sequenced a 975-bp fragment of the above murine \(PMCA1\) promoter and have defined two VSMC-specific transcription initiation sites located between a pair of \(Myb\)-binding sites: \(Myb\)-binding site-1 and site-2 at positions +440 and +528, respectively (numbering is relative to the neural cell-specific transcriptional start site). We used ribonuclease protection assays, gel shift, and luciferase reporter assays to delineate the function of these two \(Myb\)-binding sites in the mouse \(PMCA1\) promoter. Our experiments involved restriction digested deletants, single substitution point mutants in one or both \(Myb\)-binding sites, and effector constructs expressing either c-Myb, a dominant negative form of c-Myb, or an anti-Myb neutralizing antibody. Our studies show that the repression of the murine \(PMCA1\) gene at the G1/S interface of rodent VSMC requires both \(Myb\)-binding sites flanking the two transcriptional initiation sites.

MATERIALS AND METHODS

DNA Constructs—The reporter plasmid pPM1-luc and its mutants are described below. p3009, the full-length murine c-Myb expression construct, was a kind gift of Dr. Michael Kuehl, Bethesda, MD (25). The \(\Delta 5\)-myb, a dominant negative c-Myb mutant lacking amino acid residues 109 to 185 which constitute the major portion of the c-Myb DNA-binding domain, has been described elsewhere (10). psFV23, a mammalian expression plasmid encoding a single chain neutralizing antibody raised against the transactivation domain of c-Myb, was a generous gift of Dr. D. T. Curiel and Dr. K. Kasono, Birmingham, AL (26).

Cell Culture and Cell Cycle Synchronization—An optimal number (differing according to the size of the culture vessel) of SVE cells (ATCC number CRL-2018) were seeded and allowed to attach overnight in Dulbecco’s modified Eagle’s medium (10% fetal bovine serum, and 200 \(\mu\)g/ml G418 at 37 °C and 5% CO\(_2\)). Cells were washed twice with phosphate-buffered saline and incubated in Dulbecco’s modified Eagle’s medium containing 0.25% fetal bovine serum for 48 h at which point the cells were either harvested (G\(_1\) stage cells), or the medium was replaced with Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum for 8 (G\(_2\) stage cells), 16 (G\(_2\)/M stage cells), or 24 h (G/M stage cells) prior to harvest. The degree and extent of cell cycle synchronization, as assessed by DNA quantitative flow-cytometry, was identical to previously published reports (data not shown) (6, 9, 10). Primary VSMC were isolated by the method of Cornwell and Lincon (27) using 10–12 aortas from C57Bl6 mice (Charles River Laboratories, Inc., Wilmington, MA). They were then cultured and synchronized for cell cycle stages as described above.

Nuclear Run-on—Run-on assays were carried out according to Nevins (28). Nuclei were isolated from cell cycle synchronized cultures at the \(G_{i}\) and \(G_{s}\)/S stages by employing 0.3% Nonidet P-40 for cell lysis. Nascent RNA was labeled for 10 min at 37 °C, extracted with the RNeasy kit (Qiagen), DNased, and re-extracted with RNeasy before RQPCR was employed as Hot start PCR was employed as follows: 95 °C for 5 min (Pre-PCR); 35 cycles of 94 °C for 1 min, and 72 °C for 1 min; and a final extension of 72 °C for 10 min. The 975-bp PCR product was gel eluted and blunt ligated into SmaI-cut plpc3Basic (Promega) to generate the plasmid pPM1-luc. The pPM1-luc insert was sequenced on an automated sequencer (ABI Prism Model 377) using vector-specific primers Giprimer2 and Rvprimer3 (29) as well as an in vitro based primer (corresponding to position 359–410 in GenBank accession number U16707) and contig alignment was done using Sequencer 4.0 software (Gene Codes Corp.). This sequence has been deposited in the GenBank database under accession number AF162783. 3’- and/or 5’-ends of the pPM1-luc insert were deleted via restriction endonuclease digestions, blunted, and self-ligated to generate various deletion mutants. p\(\alpha\)-Element lacks the AozII-Nhel fragment spanning +418 to +68, p\(\alpha\)-myb2 lacks the Myb site-2 containing BseEI-BglII fragment spanning +488 to +557, and p\(\alpha\)-myb+2 lacks the PmlI-BglII fragment spanning +301 to +557 which contains both Myb sites-1 and -2. All deletions were confirmed by multiple restriction digestions and sizing of digested products on agarose gels.

Site-directed Mutagenesis—Site-directed mutagenesis of one or both the \(Myb\)-binding sites in the mouse \(PMCA1\) promoter was carried out as described (29) with minor modifications (30). Briefly, uridylated single stranded pPM1-luc DNA (noncoding strand) was annealed slowly over a period of 5 h in a thermal cycler with either oligo A1-M1-AS (introduces an A to G substitution in \(Myb\)-binding site-1 at +440) or oligo AM2-AS (introduces an A to G substitution in \(Myb\)-binding site-2 at +528) or with both primers simultaneously; see Table I for primer sequences. Primer extension of the annealed templates in the presence of T4 DNA polymerase and T4 DNA ligase yielded uridylated-nonuridylated relaxed, circular hybrids which were transformed into Escherichia coli DH5aMCR competent cells and four transformants from each mutagenesis reaction were screened via sequencing. A small restriction fragment (PmlI-BglII fragment spanning +301 to +557) bearing the mutated \(Myb\)-binding site(s) was cut out of the screened mutants and was used to replace the identical restriction fragment in the wild-type pPM1-luc construct to minimize chance mutations at sites other than the \(Myb\)-binding site. This generated three point mutants, viz. p1pmyb1 (Myb site-1 mutated), p1pmyb2 (Myb site-2 mutated), and p1pmyb+2 (both Myb sites mutated). The region spanning +301 to +557 was used as the point mutagenesis template and the sequence was confirmed in an automated sequencer (ABI Prism 377) and the mutant sequences were aligned with the wild-type sequence (GenBank accession number AF162783) using Sequencer 4.0 software (Gene Codes Corp.) to confirm point mutations. Ribonuclease Protection Assays—A stably transformed mouse VSMC line was constructed by isolating mouse aortic smooth muscle cells from 10 C57Bl mice as described (27). Isolated cells were stained with smooth muscle actin antibody (Sigma) and data not shown, only smooth muscle cells were present. These primary cultures were immortalized with a retrovirus carrying the SV40 large T antigen and the G418 resistance marker as described (31) and selected for 14 days with 400 \(\mu\)g/ml G418. Total RNA was isolated from T-75 flasks containing cell cycle synchronized G\(_1\) stage mouse VSMC (5 h postserum stimulation) by using the RNaseasy kit (Qiagen) and DNase treated. Three different length probes were generated (see Fig. IA). The reverse PCR primer used to PCR clone the mouse PMCA1 promoter fragment (positions 964 to 985 in GenBank accession number U16707) was annealed to AozII-digested pPM1-luc DNA and extended with Klenow DNA polymerase in the presence of [\(\alpha\-\text{32P}\)]dTTP to generate a prematurely terminated, uniformly labeled single stranded DNA probe. Probe 1 spanned +437 to +557. Probes 2 and 3 were generated by using a recombinant version of Klenow enzyme lacking both 3’ to 5’ and 5’ to 3’ exonuclease activities (MBI Fermentas): Probe 2 arose by primer extension of the reverse PCR primer from BamHI-digested pPM1-luc (+182 to +557) and Probe 3 by extension of the antisense primer EMISA M1 (see Table I) after annealing to AozII-digested pPM1-luc (+63 to +810). All three probes were digested with restriction enzymes (AatII, BstEII, and XhoI), gel purified, and quantitated by measuring Cerenkov counts. 10\(^{6}\) pm of labeled probe was annealed with 1.25, 2.5, 5.0, and 10 \(\mu\)g of total RNA overnight at 42 °C and digested with a 200-fold dilution of the nuclelease mixture prepared according to the manufacturer’s instructions (Ambion). Protected RNA products were resolved on a 5% denaturing polyacrylamide gel with dideoxy sequencing reactions carried out with

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either the reverse PCR primer and pPM1-luc or antisense EMSA M1 and pPM1-luc to help size the RNA products.

**Transient Transfection Promoter-Luciferase Assay**—Approximately 0.5 × 10^6 rat SVE cells or mouse primary VSMC were seeded per well in 6-well dishes and allowed to attach overnight. Cells were washed twice with cold PBS-buffered saline and transfected with the standard combination of plasmids (unless otherwise stated) which consisted of 1 μg of reporter plasmid (pPM1-luc or one of its mutants), 50 ng of reference reporter plasmid (pRL-TK, carrying the renilla luciferase gene under the thymidine kinase promoter; Promega), and 1 μg of effector plasmid (where applicable) with the help of 5 μl of LipofectAMINE (LifeTechnologies, Inc.) in a 1-ml overlay of Opti-MEM (LifeTechnologies, Inc.) serum-free medium according to the manufacturer’s instructions (5 h incubation at 37 °C). The overlay was removed, the cells washed twice with phosphate-buffered saline and then allowed to recover overnight in complete medium. Cells were serum starved as described above and different transfected cell populations were stimulated with serum for 0 (G0 stage) or 16 h (G1/S stage) and harvested by scraping in 250 μl of Passive Lysis Buffer (Promega). Cell lysates were subjected to 2 freeze thaws and 20 μl (10 μl in case of primary cultures) was used to measure firefly and Renilla luciferase activity in a luminometer (Lumat LB 9501; EG&G Berthold) as per a commercially available kit protocol (Dual Luciferase Assay System; Promega).

Raw relative luminescence units were corrected for auto-luminescence of the firefly and Renilla luciferase substrates (detected in mock transfected cells which were transfected with pGem7 plasmid) and normalized for transfection efficiency by dividing by the corrected Renilla relative luminescence unit values for each sample. With both rat SVE cells as well as mouse primary VSMC, mean normalized (f/fr) relative luminescence unit values for the wild-type promoter-reporter construct (pPM1-luc) at the G0 stage (mean of at least two experiments) was set as a reference value. This reference value was then used to derive ratios for individual G0 and later stage samples (sample f/r value divided by reference value). Ratios (relative normalized relative luminescence units) derived in this way from two or more experiments were used to compute mean ± S.E. In a few experiments, where a parallel comparison with the wild-type pPM1-luc reporters activity was not performed, the G0 values of the tested constructs were used as the reference value.

**Gel Mobility Shift Assays—**G1/S stage SVE cells, as well as asynchronous cultures of a human leukemia cell line known to overexpress c-Myb (K562; ATCC number CCL-243), were used to prepare nuclear extracts as described elsewhere (32). Slight modifications to the protocol’s instructions (5 h incubation at 37 °C). The overlay was removed, the cells washed twice with phosphate-buffered saline and then allowed to recover overnight in complete medium. Cells were serum starved as described above and different transfected cell populations were stimulated with serum for 0 (G0 stage) or 16 h (G1/S stage) and harvested by scraping in 250 μl of Passive Lysis Buffer (Promega). Cell lysates were subjected to 2 freeze thaws and 20 μl (10 μl in case of primary cultures) was used to measure firefly and Renilla luciferase activity in a luminometer (Lumat LB 9501; EG&G Berthold) as per a commercially available kit protocol (Dual Luciferase Assay System; Promega).

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**Statistical Analysis—**Luciferase assay data are shown as mean ± S.E. and represent results from at least two separate experiments. Student’s t test was used to make pairwise comparisons between results. Statistical significance was defined as p ≤ 0.05.

**RESULTS**

**PMCA1 Transcription—**We previously showed in immortalized rat VSMC that steady state mRNA and protein levels of PMCA1 were decreased by approximately 50% at the G1/S cell cycle stage as compared with at G0 (9). In the present study we performed numerous nuclear run-on assays in the same cell type to determine whether this 2-fold regulation was occurring at the transcriptional level. These attempts showed either no change in the rate of new PMCA1 message transcription from G0 to G1/S or a slight transcriptional repression of 30% in some experiments (mean G0 to G1/S ratio of 1 (± 0.07) 0.75 (± 0.05)).

Why some experiments showed no reduction in PMCA1 transcription may be explained by the poor sensitivity of nuclear run-on assays for detecting small (i.e. 2-fold) changes (34, 35). While the overall observed reduction in the rate of PMCA1 transcription at G1/S as compared with G0 was small, it remains possible that this decrease is sufficient to account for the 2-fold lowering of steady state mRNA levels that is known to occur. Accordingly, we proceeded to examine the structure and function of the PMCA1 gene promoter in the context of cell cycle progression in rodent VSMC.

**Wild-type Mouse PMCA1 Promoter Sequence**—A 975-bp region of the mouse PMCA1 promoter, spanning position −418 to +557 (numbering based on the most 5’ transcriptional start site mapped in mouse neural cells; see Fig. 1A) was amplified from C57Bl/6 mouse genomic DNA and sequenced. Alignment of opposing strand sequences generated via vector-specific primers (see “Materials and Methods”) showed only 5 nucleotide differences. These were clustered in the region from +78 to +138 (nucleotides 496 to 556 in the 975-bp insert) and likely arose from gel resolution ambiguities in the longest chain terminated products derived from vector-specific primers. Indeed, 4 of these differences were resolved by sequence generated from insert-specific primers (see “Materials and Methods”) and the last was resolved by comparison with the sequence reported by Du et al. (24) (GenBank accession number U16707).

By contrast, when the mouse PMCA1 promoter sequence deduced in our lab (GenBank accession number AF162783) was aligned with the sequence reported earlier (GenBank accession number U16707), there were 54 positions in which the two sequences differed and most of these were clustered in the middle of the insert. In our opinion, these could have resulted from a combination of causes, viz. differences in the mouse strains used to clone each sequence and gel resolution ambiguities in the longest chain terminated products characterized by Du et al. (24).

**Mapping the Site of Transcription Initiation—**Ribonuclease protection assays were used to definitively map the transcripts arising from the mouse PMCA1 gene in a stably transformed mouse VSMC line. As shown in Fig. 1B, when a 121-nucleotide probe covering positions +437 to +557 is used, two main transcripts are protected which map to +475 and +485, just downstream of Myb site-1 (+440). Similarly, when another probe spanning +182 to +557 is used, the same two transcripts (initiated at +475 and +485) are protected (data not shown). No transcripts are protected when a probe spanning −63 to +460 is used.

In mouse neuroblastoma cells (24), four PMCA1 transcription start sites occur (+1, +27, +47, and +64) and these appear to be specific to neural tissue. Our ribonuclease protection data show those neural-specific transcription initiation sites to be absent in PMCA1 transcripts of VSMC; instead, two smooth muscle-specific transcription initiation sites were found 475 and 485 bases downstream of the most 5’ neural-specific start site. The authenticity of these two transcription initiation sites is further bolstered by additional evidence presented below: deletion of the region between the two Myb sites (as in the deletant (pα-myb+2)) leads to a 60% drop in transcription levels from the PMCA1 promoter.

**Transient Transfection Luciferase Assays—**We next employed transient transfection of promoter-luciferase expression constructs to evaluate the importance of the two Myb-binding sites and the core promoter element in the functioning of the
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Myb site-1 is located at 528 to 533. Based on this analysis, three promoter deletion mutants were generated: pPt-myb1 with a deletion spanning 301 to 475; pPt-myb2 carries a single point mutation in Myb binding site-1 (+440); and pPt-myb1+2 carries the same single substitutions in both Myb-binding sites (see Fig. 4 and Table I).

As detailed under “Materials and Methods,” the mean G0 luciferase activity of the wild-type promoter-luciferase construct was used to normalize values of all other variants of this promoter at both G0 and G1/S cell cycle stages. Of note, the G0 value itself for the wild-type promoter was always within 10% of the mean value (Figs. 2-4). The wild-type PMCA1 promoter-reporter construct, pPM1-luc, showed a 50% reduction in luciferase activity at G1/S as compared with G0 (G0 versus G1/S = 0.99 ± 0.09 versus 0.46 ± 0.01; p = 0.03; Fig. 2). Deletion of a core promoter element defined in neural cells (p4.5′-element) did not change the repression seen at G1/S (p4.5′ Element G0/S = 0.41 ± 0.07; p = 0.04; Fig. 2). Furthermore, persistent luciferase activity in the absence of the core promoter element defined in neural cells argues for the presence of additional promoter elements active in VSMC (p4.5′ Element G0/S = 0.99 ± 0.09 versus 0.99 ± 0.09; p = NS; Fig. 2). Importantly, deletion of the restriction fragment bearing Myb site-2 (pΔ-myb2) completely abrogated the G1/S stage repression of PMCA1 promoter activity (pΔ-myb2 G0/S = 1.40 ± 0.32 versus 1.09 ± 0.19; p = NS; Fig. 2). This result supported the importance of Myb binding site-2 in bringing about G1/S-associated repression of PMCA1. However, deletion of the restriction fragment bearing both Myb site-1 and Myb site-2 (pΔ-myb1+2; lacking the region from +301 to +557) produced a 2-fold down-regulation of PMCA1 promoter-dependent luciferase activity at the G0 stage which persisted even at G1/S (pPM1-luc G0/S versus pΔ-myb1+2 G0/S = 0.99 ± 0.09 versus 0.39 ± 0.03; p = 0.02; Fig. 2). This finding supported the presence of a VSMC-specific promoter element active in VSMC in the region spanning +301 to +557. The two PMCA1 transcripts mapped from mouse VSMC are also located within this deleted region (at +475 and +485). The alternative possibility that Myb site-1 is involved in promoting basal levels of PMCA1 expression was refuted by using point mutants as described below.

Co-transfections with either wild-type or dominant negative c-Myb expression constructs were next used to increase or decrease functional c-Myb levels in VSMC transfected with the wild-type PMCA1 promoter-reporter construct (Fig. 3). c-Myb over-expression reduced G0 stage PMCA1 promoter activity by 60% and G1/S stage PMCA1 transcription by 75% as compared with the G0 stage cells transfected with pPM1-luc alone (pPM1-luc G0/S versus pΔ-myb1+2 G0/S = 0.99 ± 0.09 versus 0.39 ± 0.03; p = 0.02; Fig. 3). Expression of the dominant negative c-Myb mutant completely relieved the G1/S stage repression of luciferase activity in a dose dependent manner, with higher doses of the mutant producing greater derepression. A mammalian expression construct encoding an anti-c-Myb single chain antibody has recently been shown to inhibit the transactivation activity of c-Myb (26). When this anti-c-Myb antibody construct was co-transfected into SVE...
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TABLE I
Oligodeoxynucleotides

| Name and description | Primer sequence |
|----------------------|-----------------|
| ΔM1-AS, anti-sense sequence for Myb-binding site 1 (+440) with point mutation | 5′-AGGGGGCTTACCAGGCTGCTGGTG-3′ |
| ΔM2-AS, anti-sense sequence for Myb-binding site 2 (+528) with point mutation | 5′-AGTCTACAGGGGCAGCCATCTGCGGG-3′ |
| EMSA M1, double stranded oligo harboring Myb-binding site 1 (+440) | 5′-GGAGGCCTCCGAGCCAGGTCGGCAGTCCGAGG-3′ |
| 3′-CCTCGCAGCTGTCGGACCG-5′ | 5′-CCTCCCG-3′ |
| EMSA Pt-M1, double stranded oligo harboring single point mutation of Myb-binding site 1 (+440) | 5′-CTGGGACCAGCAGCTGTCGGACCG-3′ |
| EMSA M2, double stranded oligo harboring Myb-binding site 2 (+528) | 5′-GCCGAGCAGCTGTCGGACCG-3′ |
| EMSA Pt-M2, double stranded oligo harboring single point mutation of Myb-binding site 2 (+528) | 5′-CCCCGGAGCAGCTGTCGGACCG-3′ |

![Fig. 2. Transient transfection luciferase assays with wild-type and deleted PMCA1 promoters.](http://www.jbc.org/)

**Fig. 2.** Transient transfection luciferase assays with wild-type and deleted PMCA1 promoters. The 975-bp mouse PMCA1 promoter was ligated upstream of the firefly luciferase cDNA to make the construct labeled pPM1-luc. The luciferase vector without any upstream promoter was termed “Promoterless.” Δ-myb2 has been deleted of a restriction fragment spanning +488 to +557 and lacks Myb site-2. Δ-myb1-2 lacks a fragment spanning +301 to +557 which contained both Myb binding sites-1 and -2. The Δ-5′ element lacks a promoter region known to be active in murine neural cells (24). The constructs were used to transiently transfet rat VSMC with luciferase activities being measured at G0 and G1/S stages. Values were corrected for background luminescence as well as transfection efficiency and normalized to the mean luciferase activity of the wild-type promoter at the G0 stage (see “Materials and Methods”). Data shown are the mean ± S.E. of at least two experiments.

Table 1

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Single and double stranded nucleotide sequences employed in the various experiments. Position numbers correspond to GeneBank accession number AF162783. Nucleotides shown in bold and underlined represent the Myb consensus DNA binding sequence. Nucleotides shown in lower case represent substitution mutations.

To test the functional specificity of our point mutants, we co-transfected pPT-myb1-2 with the anti-Myb encoding construct. Reduction in functional c-Myb activity had no effect on reporter levels which confirmed that the double point mutant had lost c-Myb responsiveness (data not shown). Finally, the alleviation of G1/S-associated PMCA1 repression was also observed when the point mutants were tested in primary cultures of mouse VSMC (data not shown).

**Gel Shift Studies**—In order to determine whether putative Myb-binding sites-1 and -2 of the murine PMCA1 promoter can actually bind c-Myb protein, we next employed the gel shift assay (Fig. 5). Nuclear extracts from a human leukemic cell line (K562 cells) known to express high levels of c-Myb (33) were used for gel shift assays with end-labeled Myb site-1 or Myb site-2 double stranded oligonucleotides. Increasing amounts of the nuclear extract led to the formation of higher amounts of c-Myb-DNA complexes. Excess unlabeled Myb site-1 oligo successfully competed with labeled Myb site-1 DNA for binding to c-Myb while excess unlabeled mutant Myb site-1 oligo bearing a single point mutation did not (Fig. 5A). Myb site-2 double stranded oligo showed similar results. An excess of unlabeled Myb site-2 oligo could out-compete labeled Myb site-2 oligo in binding to c-Myb while an excess unlabeled mutant Myb site-2 oligo could not (Fig. 5B). We also carried out gel shift assays using nuclear extracts made from rat SVE cells at the G1/S stage. Although these experiments always yielded results identical to the ones obtained with extracts from the human cell line, the intensities of the shifted bands were always weaker (data not shown). This finding is consistent with the markedly lower amounts of c-Myb in rat VSMC as compared with K562 cells, and argues against nonspecific binding of non-Myb proteins to the target oligos.

**DISCUSSION**

Results of numerous studies have revealed that the c-Myb transcription factor regulates [Ca^{2+}], during the G0 to S phase cell cycle progression of VSMC and fibroblasts (6, 7, 9–11, 39). Overexpression of wild-type c-Myb has been shown to increase [Ca^{2+}], at both the G0 and G1/S cell cycle stages as compared with control transfected cells (10), while the use of dominant negative c-Myb mutants demonstrated that reductions in c-Myb activity abolished the normal rise in both resting and stored [Ca^{2+}], as cells moved to the G1/S transition (10). This latter effect was due to a marked increase in the Na^+-independent Ca^{2+} efflux rate, and was associated with important reductions in the rate of S-phase entry and cell proliferation.
It was subsequently shown that c-Myb activity levels are inversely related to mRNA and protein levels of PMCA1 (9), which normally show a ~50% reduction at G1/S as compared with G0. Overexpression of PMCA1, independent of any manipulations in c-Myb activity, also resulted in increased rates of Ca\(^{2+}\) efflux and significant reductions in [Ca\(^{2+}\)]\(_{i}\) during G1/S progression and proliferation (9). Thus, abolition of the normal repression of endogenous PMCA1 expression at G1/S, by either dominant negative c-Myb constructs or overexpression of a transfected PMCA1, prevented the normal fall in the Ca\(^{2+}\) efflux rate and disabled the accumulation of intracellular Ca\(^{2+}\) (9, 10). Together, the above data led to our definition of the PMCA1 Ca\(^{2+}\) efflux pump as an end-effector of the c-Myb-dependent elevation in [Ca\(^{2+}\)]\(_{i}\) critically required for G1/S transitions in VSMC.

Previous investigations into PMCA1 gene regulation have suggested the existence of agonist- and tissue-specific signaling pathways (23, 24, 40). Phorbol ester or angiotensin II treatments of non-synchronized rat VSMC were shown to produce protein kinase C-dependent increases in PMCA1 mRNA (8–20-fold) and protein (3–4-fold) within 4–6 h of agonist treatment (23). A protein kinase A-dependent pathway was implicated in both cAMP- and thapsigargin-induced increases in PMCA1 expression in non-synchronized endothelial cells derived from resistance vessels of the rat brain (40). Of interest, rat aortic endothelial cells did not show increases in PMCA1 levels in response to these latter agents (40). Non-synchronized mouse neuroblastoma cells also responded to a 4-h treatment with phorbol ester by exhibiting a 5-fold increase in steady state PMCA1 mRNA (24). Importantly, the regulation of this response appeared to depend on transcriptional activation of the PMCA1 gene via a core promoter segment (~2442 to ~1169) of the murine PMCA1 gene (24). When cell cycle-synchronized rat VSMC are studied, the early stages of G1/S progression (i.e. 0.5, 1, 2, and 4 h post-serum stimulation) also show significantly increased levels of PMCA1 mRNA as compared with either G0 or G1/S levels (9). Of interest, these elevations in PMCA1 expression are coincident with the expression of early-response genes and with known mitogen-mediated elevations in [Ca\(^{2+}\)]\(_{i}\) (41). The above studies suggest that a variety of signal transduction pathways, early response genes, and/or transient increases in [Ca\(^{2+}\)]\(_{i}\) during the early part of G0 to G1 progression may act to increase PMCA1 expression. However, the subsequent down-regulation of PMCA1.

**Fig. 3.** Transient transfection luciferase assays with overexpression of wild-type or dominant negative c-Myb constructs. pPM1-luc was used to transiently transfect rat VSMC either alone or in combination with expression constructs for wild-type murine c-Myb, a dominant negative c-Myb mutant (ΔS-Myb), or an anti-Myb neutralizing antibody (anti-Myb Ab). Luciferase activities were measured at the G0 and G1/S stages as described under “Materials and Methods.” Data shown are the mean ± S.E. of at least two experiments.

**Fig. 4.** Transient transfection luciferase assays with point mutants of the mouse PMCA1 promoter. Single nucleotide substitutions were made in either the first, second, or both Myb-binding sites of the mouse PMCA1 promoter ligated upstream of the firefly luciferase cDNA to produce three point mutants, viz. Pt-myb1, Pt-myb2, and Pt-myb1+2, respectively. These point mutants were used to transfect rat VSMC and luciferase activity was measured at the G0 and G1/S stages. Data shown have been corrected for background luminescence and transfection efficiency and normalized to the mean luciferase activity of the wild-type promoter at the G0 stage. Data shown are the mean ± S.E. of at least two experiments.
levels and PMCA1-mediated Ca\(^{2+}\) efflux rates at the G\(_1\)/S interface (9) requires an opposing effect on PMCA1 expression at this point in the cell cycle. The present study has elucidated a molecular mechanism through which c-Myb mediates this opposing effect.

While it has recently been shown that c-Myb activity can decrease the half-life of thrombospondin 2 mRNA in mouse fibroblasts, the mechanism through which c-Myb mediated this effect was not elucidated (2). Bein et al. (2) have speculated that c-Myb may transactivate the expression of a labile ribonuclease which in turn could account for the enhanced degradation of thrombospondin 2 mRNA. Although a c-Myb-dependent reduction in the stability of PMCA1 mRNA could conceivably contribute to the down-regulation of steady state PMCA1 mRNA levels at the G\(_1\)/S interface, the findings of the present study suggest a transcriptional mechanism of PMCA1 regulation. Our nuclear run-on data show at least a 25% decrease in the rate of PMCA1 transcription at G\(_1\)/S as compared with G\(_0\) (data not shown). As it is difficult to document 2-fold differences in transcription rates with nuclear run-on assays, the above result is likely an underestimate of the reduction in PMCA1 transcription.

Using MatInspector V2.2 (36), our analysis of the human PMCA1 gene sequence reveals two putative c-Myb-binding sites at position +888 and +1182. Indeed, a comparison of the human and murine PMCA1 promoter regions has revealed several other similarities as well (16). Both genes share features such as a large first intron (20–30 kilobases), a translational start in exon 2, and the presence of a cryptic promoter in the remaining 5'-flanking region within this mutant construct. These reports earlier (24), suggest the presence of two promoters in the mouse PMCA1 gene: one element (spanning −256 to −118) active in neural cells and the other (between +301 to +485) active in VSMC.

The preceding discussion has shown that the presence of multiple promoter elements with distinct transcriptional start sites is possible in the human PMCA1 gene and highly probable in the murine gene. This is analogous to the recent realization from transgenic mouse studies that multiple, independent, cis-regulatory modules are required to direct the developmental expression of muscle-specific genes. More specifically, a gene may have one promoter for skeletal muscle expression, another for cardiac expression, and one or more for expression in smooth muscle cells of various types (53). The tissue-specific activation of each promoter element would then be accomplished by unique sets of transcription factors. Indeed, this type of combinatorial regulation of gene expression appears to be a general strategy for tissue-specific expression in other genes as well (54, 55).

Several genes involved in cell proliferation are known to be transcriptionally activated by c-Myb. These include c-nyc (56, 57), cd2 (58), topoisomerase IIa (59), DNA polymerase α (60), c-kit (61), and the gene for proliferating cell nuclear antigen (PCNA) (62). In addition, c-Myb is also known to repress genes such as c-erbB-2 (63), the mouse N-ras promoter as tested in avian fibroblasts (64), the monocytic gene MR1P4 in HL-60 cells (65), the 5-lipoxygenase gene during HL-60 differentiation (66), the c-kit gene in non c-Kit-expressing cells (67), and the promoter function of both human and mouse c-fms genes (68). Of the above six genes known to be negatively regulated by c-Myb, four are cytoplasmic proteins with roles in cell-cell...
interactions or cell differentiation, while two are proto-oncogenes encoding transmembrane proteins involved in signal transduction (c-erbB2 and N-ras).

The present study highlights similar consequences of c-Myb activity in rodent VSMC, i.e. control of a transmembrane protein involved in cell proliferation via transcriptional repression. Our studies conclude that the PMCA1 pump is a physiologically important c-Myb-responsive cell proliferation gene over which c-Myb mediates cell cycle-specific negative regulation through specific c-Myb binding sites in the PMCA1 promoter. The c-Myb-mediated down-regulation of PMCA1 levels allows sustained elevations in resting and releasable [Ca^{2+}]_i at the G_1/S interface (9), which in turn facilitate G_1 to S cell cycle transitions through a variety of putative Ca^{2+}-responsive effectors (9, 41).

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Talat Afroze and Mansoor Husain

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