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Regulation of Expression of the Growth-State-Related Genes 2F1 and 2A9 During Entry of Quiescent Smooth Muscle Cells Into the Cell Cycle

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Abstract Vascular smooth muscle cells (SMCs) play a key role in the development of major arteries. Furthermore, abnormal growth of vascular smooth muscle cells has been implicated in the progression of major diseases of the cardiovascular system. Here, we report detection in primary cultures of bovine vascular smooth muscle cells of mRNA for two growth-state-related genes, 2F1 and 2A9, which code for a mitochondrial ADP/ATP carrier and calcyclin, respectively, and on the characterization of their cell cycle expression. Cultures of exponentially growing smooth muscle cells were made quiescent by serum deprivation. Upon readdition of serum, cells entered the cell cycle synchronously; DNA synthesis began 12 h post-serum addition. Levels of 2F1 and 2A9 RNA were low in quiescent cells and increased between 2 and 4 h post-serum addition. No changes in the rates of transcription of the 2F1 or 2A9 genes were detected by nuclear run-off assays during the time course. Thus the regulation of changes in expression of 2F1 and 2A9 in early G1 is mediated post-transcriptionally.

Key words: SMCs, mRNA, mitochondrial ADP/ATP carrier, calcyclin, cell cycle

The major cellular component of an artery resides within the medial layer and is composed predominantly of smooth muscle cells (SMCs). Atherosclerosis has been shown to involve aberrant SMC growth. Following an initiating event, migration of the SMC from the medial layer to the intima of an artery leads to abnormal SMC proliferation within this environment. Furthermore, evidence suggests that the SMCs within such a lesion are not normal, and that they may be partially transformed (Benditt and Benditt, 1973; Penn et al., 1986).

Two categories of genes have been identified to be involved in the growth of mammalian cells, the proto-oncogenes and growth-state-related or cell division cycle (cdc) genes. In earlier work, we characterized the SMC expression of the nuclear proto-oncogenes *c-fos* and *c-myc* (Kindy and Sonenshein, 1986) as well as of *c-myb*, an oncogene not known previously to be synthesized by the SMC (Reilly et al., 1989; Brown, Kindy and Sonenshein, manuscript in preparation). Here we have examined genes whose expression relates to the growth state of the cell. Many of these growth-state-related or growth-regulated genes have been isolated by differential hybridization techniques (Cochran et al., 1983). Baserga and co-workers (Hirschhorn et al., 1984) employed a temperature-sensitive mutant of baby hamster kidney cells (BHK) to isolate cDNAs whose expression varied specifically during the G1 phase of the cell cycle. For two of these clones, 2F1 and 2A9, they noted that expression was low during quiescence and increased early in G1. Subsequently DNA sequencing and homology analysis demonstrated that the 2F1 gene encodes a mitochondrial ADP/ATP translocase (Battini et al., 1987) and that the 2A9 gene has a putative calcium binding domain, and was therefore given the name calcyclin (Ferrari et al., 1987); homology of 2A9 with the feline sarcoma virus transforming gene (*fes*) has also been noted (Hirschhorn et al., 1984). Deregulation and overexpression of 2A9 have been detected in acute

Abbreviations used: FCS, fetal calf serum; SMC, smooth muscle cell.

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myeloid leukemia, suggesting its involvement in cell cycle progression (Calabretta et al., 1986). Here we have demonstrated the expression of the 2F1 and 2A9 genes in cultured calf vascular smooth muscle cells. We show that the increased expression of the mRNA for these genes during the early G1 is regulated post-transcriptionally.

MATERIALS AND METHODS

Cell Isolation and Culture Conditions

Smooth muscle cells were explanted from the medial layer of the aortic arch of female calves and grown in tissue culture, as described previously (Stepp et al., 1986). Cells were grown in first passage for 1 week prior to subsequent trypsinization and repassage. All experiments were conducted on second- and third-passage cells. Tissue culture reagents were purchased from Grand Island Biological Co.

Cells were plated at an initial density of 5×10^5 cells per P150 dish. These subconfluent cultures were incubated for 3 days in medium supplemented with 10% fetal calf serum (FCS) to permit exponential growth. They were then incubated in medium supplemented with 0.5% FCS and 10 mM HEPES, pH 7.3, to deprive them of serum growth factors, for an additional 3 days. The cells were then stimulated by addition of fresh medium containing 15% FCS. DNA synthesis within the cultures was assessed during a 1-h pulse period with [^{3}H]thymidine (2 μCi/ml; 80 Ci/mmol; Amersham Corp.) by using autoradiography to determine the percent labelled nuclei, as described previously (Kindy and Sonenshein, 1986).

RNA Hybridization Analysis

Total RNA was isolated by the procedure of Chirgwin et al. (1979). Equal quantities (15 μg) of RNA were subjected to electrophoresis on 1.0% agarose-formaldehyde gels and resulting Northern blots were hybridized and washed as described previously (Dean et al., 1983).

Cloned DNAs

Probes employed were as follows: 2F1, baby hamster kidney cDNA clone p13-2F1 or the Eco RI–Sal I fragment of p13-2F1 (Hirschhorn et al., 1984) and human cDNA clone hp2F1 (Battini et al., 1987); 2A9, baby hamster kidney cDNA clone p13-2A9 or Eco RI–Sal I fragment of p13-2A9 (Hirschhorn et al., 1984) and human cDNA clone 2A9 (Ferrari et al., 1987); β-actin, a rat β-actin cDNA which contains coding information capable of cross-hybridization with γ-actin (Bond and Farmer, 1983); histone H3.2, pRAH3.2 (Alterman et al., 1984). For Northern blotting, either whole plasmids or isolated fragments were radiolabelled by the random-primer procedure of Feinberg and Vogelstein (1982) (10^9 cpm/ug of DNA). Entire plasmids were used for transcriptional analysis.

Nuclear Run-Off Transcriptional Analysis

Nuclei were isolated and analyzed for run-off transcripts by the method of Greenberg and Ziff (1984). Briefly, 1×10^7 nuclei were incubated in the presence of [^{32}P]UTP (150 μCi, 3,200 μCi/mM; ICN) for 30 min during which time the incorporation of radioactivity was linear. RNA was isolated and equal counts/minute (4.5×10^6) were hybridized to plasmid DNA (10 μg/slot) immobilized on nitrocellulose paper as described previously (Levine et al., 1986).

RESULTS

Synchronization of Smooth Muscle Cell Cultures

The serum deprivation/stimulation protocol outlined in Materials and Methods was used to synchronize SMC cultures. As described previously (Kindy and Sonenshein, 1986), DNA synthesis and the presence of histone H3.2 mRNA, an S-phase expressed gene, were routinely monitored to follow changes in the growth state. Incorporation of [^{3}H]thymidine into nuclei was used to determine the percent labelled nuclei as a measure of DNA synthesis within individual cells. Cultures of exponentially growing cells demonstrated approximately 50% labelled nuclei, while only 1–2% of cells deprived of serum for 72 h were labelled (Table I). Stimulation of cultures with serum resulted in an increase in labelled nuclei by 12 h, indicating the beginning of DNA synthesis; percent labelled nuclei values peaked at 20 h with 95% of cells incorporating [^{3}H]thymidine (Table I). Similarly, histone H3.2 mRNA was undetectable in cells deprived of serum for 72 h; levels increased at 12 h, and peaked between 16 and 20 h, parallelling the elevation in [^{3}H]thymidine incorporation (data not shown). (In agreement with these findings are cytofluorimetric measurements, performed on similarly treated cultures, as described previously (Kindy and Sonenshein, 1986).) Thus cells

TABLE I. DNA Synthesis in Aortic Smooth Muscle Cell Cultures

Hours after serum addition	% labelled nuclei
0	1
0.25	—
0.5	—
1	—
2	1
4	—
8	1
12	24
16	85
20	95
24	32

are quiescent after 72 h of serum deprivation. Following serum addition, the cells exit G0 and transit through the G1 phase; DNA synthesis begins at 12 h and peaks between 16 and 20 h.

Expression of Cloned Growth-State-Related Genes

In order to characterize the expression of the two growth-state-related genes, 2F1 and 2A9, in SMCs, total RNA was prepared from cells during exponential growth and quiescence and at 2 and 18 h following serum stimulation. As shown in the Northern blot in Figure 1a, 2F1 mRNA levels were elevated in exponentially growing compared to quiescent cells. The mRNA for 2F1 is approximately 1.5 kb. Stimulation of quiescent SMCs with 15% FCS resulted in a small but consistent increase in 2F1 mRNA levels by 2 h, but a more dramatic increase at 18 h. A closer analysis of the changes in 2F1 mRNA levels (Fig. 1b) indicated that the major increase occurred between 2 and 4 h after stimulation. After this increase, the RNA levels remained elevated for 16 to 18 h, and showed a slight decline a few hours later, by 20 to 24 h after serum stimulation.

The analysis of 2A9 gene expression is illustrated in Figure 2. The pattern of changes is similar to that observed with 2F1 gene expression. The level of the 0.75-kb 2A9 mRNA is low in quiescent cells. A significant increase occurs between 2 and 4 h after serum stimulation, and the levels remain elevated during S phase. Thus gene expression of both 2F1 and 2A9 is low in quiescent SMCs, and increases early during the G1 phase of the cell cycle.

Post-Transcriptional Regulation of Changes in Gene Expression

To evaluate the transcriptional component of the changes in 2F1 and 2A9 gene expression, nuclei were isolated from cells during exponential growth and quiescence and 2, 4, and 18 h after serum stimulation. Gene transcription was examined by nuclear run-off assays (Fig. 3). No significant change in the rate of 2F1 or 2A9 gene transcription was detected over the time course. Similar results were obtained with cloned human genes (Battini et al., 1987; Ferrari et al., 1987) (data not shown). In contrast, transcription of the β-actin and histone H3.2 genes, added as controls, varied as expected based on our previous observations (Kindy and Sonenshein, 1986). Histone gene transcription is lower in quiescent than exponentially growing cells, and increases significantly as the cells enter S phase. Actin gene transcription is also lower in quiescent than in exponentially growing cells; it increases by 2 h after serum stimulation and again as the cells enter S phase. Thus the failure to observe increased transcription of 2F1 and 2A9 with cell cycle transitions indicates that the regulation of changes in mRNA expression of these genes does not occur at the level of transcription.

DISCUSSION

The 2F1 and 2A9 genes are expressed in cultured bovine vascular SMCs; changes in their expression occur in a cell-cycle-dependent fashion and are controlled post-transcriptionally. Increases in 2F1 and 2A9 mRNA levels occur between 2 and 4 h following addition of serum to cultured quiescent SMCs. Baserga and co-workers noted that expression of these genes in BHK cells increased by 6 h, the earliest time point studied (Hirschhorn et al., 1984). Thus these genes are markers for progression into early G1. Recently, the promoter region of the human calcyclin gene has been isolated and characterized (Ferrari et al., 1987; Ghezzo et al., 1988). Using transient CAT expression assays in transfected BALB/c/3T3 cells, Ghezzo et al. (1988) identified an enhancer, a negative control region responsive to EGF, and a positive regulatory element stimulated by FCS and platelet-derived growth factor (PDGF). Their results indicate that FCS activates promoter transcription between two- to 3.5-fold, compared to levels in quiescent cells. This contrasts with our finding

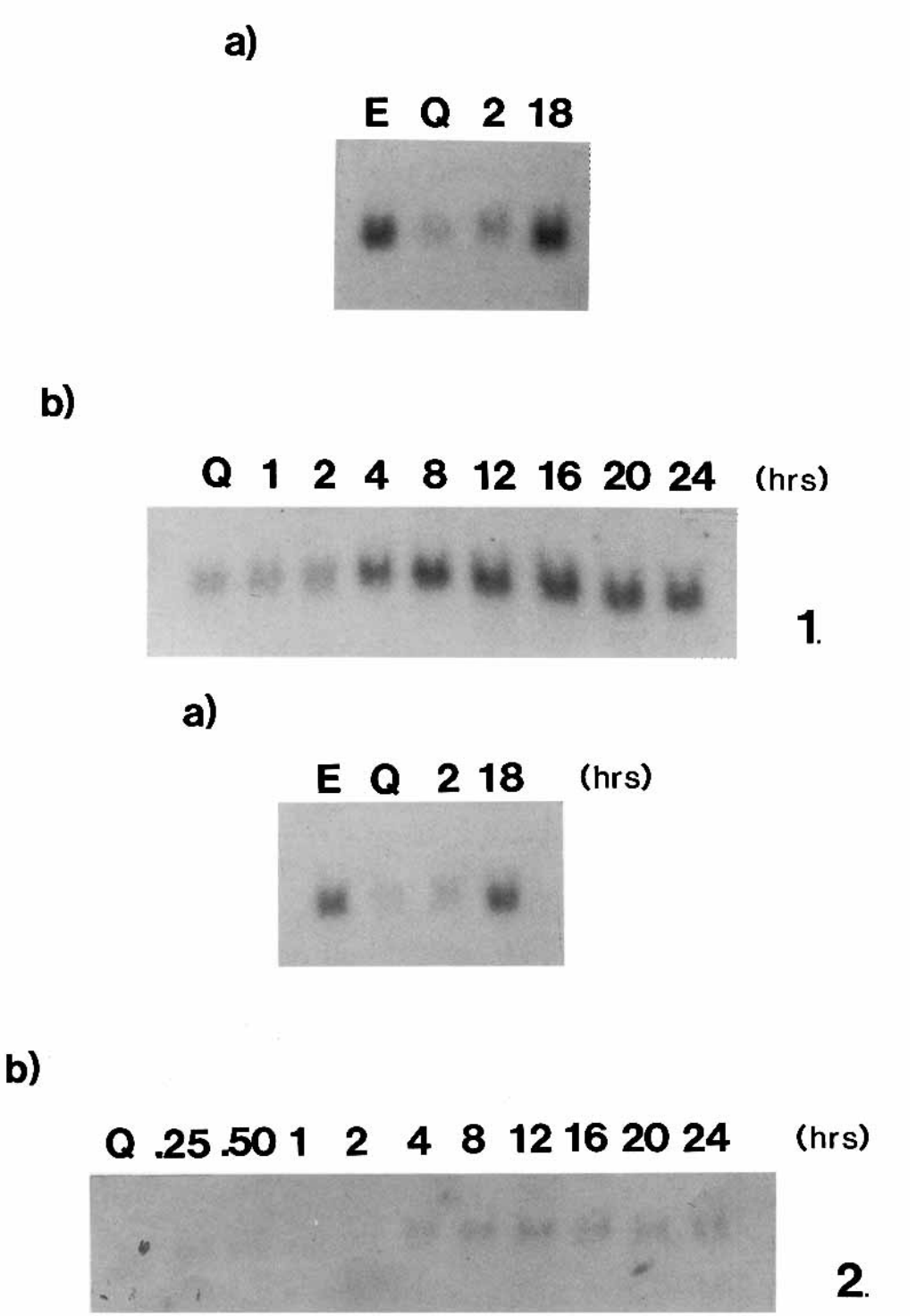

Fig. 1. Expression of the 2F1 gene during the transition from queiscence to S phase. Total RNA was extracted at the various times (E, exponential; Q, quiescent) and samples (15 μg) were subjected to Northern blot analysis by using the 2F1 probe (insert of the p13-2F1 clone). (The apparent shift in 2F1 RNA mobility was not reproducibly observed and presumably results from a gel aberration.)

Fig. 2. Expression of the 2A9 gene during the transition from quiescence to S phase. Total RNA, isolated at the indicated times (E, exponential; Q, quiescent), was subjected to Northern blot analysis by using the 2A9 probe (insert of the p13-2A9 clone).

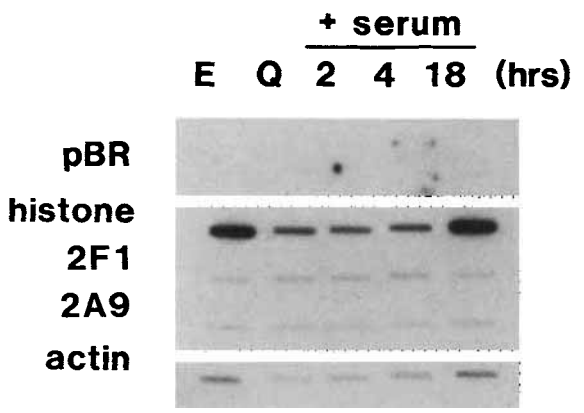

Fig. 3. Nuclear run-off analysis of gene transcription during transition from quiescence to S phase. Nuclei were isolated from cells growing exponentially (E), in quiescence (Q), and at 2, 4 and 18 h following serum stimulation and subjected to run-off transcription analysis.

that transcription does not change during the transition from quiescence to G1. This difference may be due to the method of measuring transcription. Alternately, the two different cell systems may have different mechanisms for control of 2A9 mRNA levels, as has been seen for *c-myc* gene expression (Kindy and Sonenshein, 1986; Greenberg and Ziff, 1984).

A pathway of cell cycle changes in gene expression within SMCs is beginning to emerge from this and previous work (Kindy and Sonenshein, 1986; Brown, Kindy, and Sonenshein, manuscript in preparation). Serum stimulation results in an immediate early response of the *c-fos* and β- and γ-actin genes (Kindy and Sonenshein, 1986). Increased mRNA levels are detected within 15 min following serum stimulation. Increases in *c-myc* mRNA are detected within 1 h of serum stimulation, and levels peak at 2 h. These modulations are followed by increases in 2F1 and 2A9 gene expression. (A second ADP/ATP translocase member of the "2F1 gene family" has recently been cloned (Houldsworth and Attardi, 1988); it is unclear whether this gene is also expressed in SMCs.) The increase in *c-myb* mRNA levels occurs in mid to late G1, prior to the onset of DNA synthesis (Brown, Kindy, and Sonenshein, manuscript in preparation) and the accompanying rise in histone H3.2 mRNA levels. These genes can now be employed as markers to help elucidate control of cell cycle progression. For example, in collaboration with C. Reilly and R. Rosenberg, we have recently used these genetic markers to analyze the heparin inhibition of SMC proliferation; heparin blocks gene expression in mid to late G1, prior to S phase (Reilly et al., 1989). Furthermore, a comparison of the expression of these genes in normal and atherosclerotic smooth muscle cells may help characterize the nature of the proliferative state of these two cell populations.

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