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Isolation of a cDNA Encoding a Growth-Arrest Associated Gene and Characterization of Its Regulation

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Abstract We are interested in understanding the molecular events associated with the growth-arrest of vascular SMCs. We constructed a subtracted cDNA library enriched in nucleotide sequences associated with quiescent SMCs. This library was screened with similarly subtracted ^{32}P-labeled cDNAs to identify growth-arrest associated cDNA clones. Characterization of 19 of these cDNA clones revealed that 9 hybridized to mRNAs that exhibited a 2–3-fold increase in growth-arrested SMCs. In addition, two other cDNAs hybridized to a 5 Kb mRNA that was elevated approximately 10-fold in high density growth-arrested SMCs. Genomic Southern blot hybridization and DNA sequencing analysis indicated that these cDNAs encoded the same gene (LG7) and that this gene may be a member of a multigene family or that it may contain a sequence shared by other unrelated genes. Augmented expression of LG7 was associated with both high cell density and serum deprivation induced growth-arrest. LG7 mRNA expression was down-regulated when SMCs were incubated with FBS or with reagents that arrest cells in early S-phase. Additional analysis with cell cycle specific inhibitors indicated that LG7 mRNA levels were also low when cells were blocked at the G_2 phase of the cell cycle but blockage at mitosis resulted in an elevated level of LG7 mRNA. We further demonstrated that the expression of LG7 was dependent on the presence of a relatively labile protein since protein synthesis inhibitors specifically blocked the expression of this mRNA but not the mRNA expression of α_1(III) collagen or ferritin H-chain. Finally, we demonstrated that Bt_2cAMP was able to induce mRNA expression of LG7 within 2 h, suggesting that this gene may be directly regulated via the cyclic-AMP-dependent protein kinase pathway.

Key words: smooth muscle cell, growth-arrest, gene expression, cAMP, butyrate, cell cycle

Unscheduled migration and proliferation of normally quiescent vascular smooth muscle cells (SMCs) in the blood vessel is considered a critical event in various human vascular pathologies [Schwartz et al., 1986; Ross, 1993]. The major emphasis has been on the role of growth factors and cytokines that stimulate these cells to undergo G_1 transition [Ross, 1993]. Considerably less attention has been focused on the pathways that maintain these cells in the G_0 state. Yet cell fusion and gene transfer experiments clearly indicate that quiescent and senescent states are predominate over the proliferative state, suggesting that certain dominant gene products must be down-regulated prior to cellular proliferation [Stein and Yanishevsky, 1981; Lumpkin et al., 1986; Padmanabhan et al., 1987]. Recently, a small group of genes have been identified that are rapidly down-regulated in response to mitogenic stimulation [Schneider et al., 1988; Bedard et al., 1989; Fornace et al., 1989; Gorski et al., 1993]. Consistent with the cell fusion and gene transfer experiments, when some of these gene products are reintroduced into cells, they are able to block cell cycle progression [Nuell et al., 1991; Del Sal et al., 1992; Zhan et al., 1994; Barone et al., 1994]. One of these growth-arrest associated genes, GADD153/CHOP, is believed to act by dimerizing with transcription factors of the C/EBP family [Barone et al., 1994]. A second growth-arrest associated gene, Gax, is a homeobox gene also believed to be involved in transcriptional regulation [Gorski et al., 1993]. These results suggest the presence of a complex but poorly understood network of genes that can maintain cells in a growth-arrested state.

Growth-arrest in vascular SMCs is specifically associated with increased expression of several extracellular matrix proteins, apolipoprotein E, ferritin H-chain, Gax, and a number of unidentified cellular and secreted polypeptides [Stepp et al., 1986; Majack et al., 1988; Liau and Chan, 1989; Liau et al., 1990, 1991; Gorski et al.,

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1993]. These results indicate that active modulation of various cellular events is associated with SMCs maintained in the quiescent state. Of particular interest is the finding that postconfluent SMCs synthesize a cell surface-associated heparan sulfate containing glycosaminoglycan that is a potent inhibitor of SMC proliferation [Fritz et al., 1985]. We previously used differential hybridization to isolate cDNAs encoding mRNAs that are expressed at an elevated level in high density growth-arrested vascular SMCs [Liau et al., 1991]. Two of these cDNAs were identified and found to encode α_2(I) collagen and ferritin H-chain, respectively. We further demonstrated that elevated ferritin H-chain mRNA level was promoted by cAMP addition and increased expression of ferritin H-chain was also associated with myogenic differentiation [Liau et al., 1991]. In the present study, we are interested in identifying additional growth-arrest associated genes in vascular smooth muscle cells. We report here the isolation of a cDNA for a potentially novel growth-arrest associated mRNA and describe its regulation in rabbit vascular SMCs.

METHODS

Materials

Defined fetal bovine serum (FBS) was purchased from Hyclone Laboratories, Inc. (Logan, UT). Bovine insulin and transferrin, Bt_2cAMP (sodium salt), 8-bromoadenosine 3′:5′-cyclic monophosphate (8Br-cAMP) (sodium salt), n-butyric acid (sodium salt), hydroxyurea, aphidicolin, etoposide, colchicine, and puromycin were purchased from Sigma (St. Louis, MO). Cycloheximide and Colcemid were purchased from Boehringer Mannheim Corp. (Indianapolis, IN).

Cell Culture

SMCs were isolated from the aorta of New Zealand white rabbits by enzymatic digestion as previously described [Liau and Chan, 1989]. They were routinely cultured in medium 199 with 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B. SMCs were made quiescent by changing to medium containing 0.5% FBS, 10 μM insulin, and 5 μg/ml transferrin (low serum media) for 48–72 h or by allowing cells to grow to high cell density (5 days postconfluent) [Liau et al., 1990]. For cDNA library synthesis, SMCs were high density growth-arrested and subsequently cultured in L.S. media for an additional 48 h. RNA used for the subtraction was obtained from preconfluent SMCs made quiescent by L.S. media and subsequently challenged with 20% FBS for 10 h. Hydroxyurea was used at a final concentration of 8 mM (48 h), aphidicolin was used at a concentration of 5 μg/ml (24 h), colchicine was used at a concentration of 8 μg/ml (48 h), etoposide was used at a concentration of 0.6 μg/ml (36 h), and Colcemid was used at a concentration of 0.2 μg/ml (36 h). Bt_2cAMP, 8Br-cAMP and sodium butyrate were added to a final concentration of 1 mM. Cycloheximide and puromycin were used at a final concentration of 2 and 30 μg/ml, respectively.

RNA Preparation and Northern Blot Analysis

Total cellular RNA was isolated using guanidinum isothiocynate/cesium chloride as previously described [Liau and Chan, 1989]. Five micrograms of total RNA was size fractionated on a 1% denaturing formaldehyde agarose gel and transferred overnight to nylon membranes (Nytran; Schleicher & Schuell, Keene, NH) in 10 × SSC. The transferred RNA was crosslinked to the membrane by UV irradiation (120 mJ) using the Stratalinker (Stratagene, La Jolla, CA). The immobilized RNA was hybridized overnight with 3–5 × 10^6 cpm/ml of ^{32}P-labeled DNA probes prepared by random primer synthesis (Boehringer Mannheim). The stringency of the hybridization and wash conditions have been previously described [Liau et al., 1985]. A 1 Kb hamster cDNA probe for GADD153 was kindly provided by Dr. A. Fornace (NIH, MD). The 638 bp ferritin H-chain cDNA and the 1.9 kb human α skeletal actin cDNA have been previously described [Liau et al., 1991]. Quantitation of autoradiograms was performed on a Hoefer GS-300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA).

Subtracted cDNA Library Construction and Screening

Twenty-two micrograms of poly(A^+) mRNA isolated from growth-arrested (G_0) SMCs was used as the template for cDNA synthesis with random primers and Moloney murine leukemia virus H^- reverse transcriptase (GIBCO/BRL Life Technologies Inc., Gaithersburg, MD). Approximately 9.5 μg of cDNA was synthesized and hybridized with 40 μg of poly (A^+) mRNA isolated from SMCs stimulated with 20% FBS for

10 h. Hybridization was carried out at 65°C for 14 h and the single stranded cDNA was separated from duplex cDNA-RNA and excess RNA by hydroxylapatite column chromatography. Approximately 80 ng of cDNA was recovered and this G_0-enriched cDNA was used as a template for second-strand synthesis using DNA polymerase I. The double stranded cDNA was blunt ended with T_4 DNA polymerase, ligated to *Eco*RI adaptors (Promega Corp., Madison, WI), and subsequently ligated to *Eco*RI digested λZAP II vector (Stratagene). This subtracted cDNA library contained approximately 3.5×10^4 individual clones, with an average insert size of about 500 bp. For screening purpose, the G_0 enriched cDNA library was plated out at a density of 8,000 plaques per 150 cm^2 dish and transferred to duplicate nitrocellulose filters. The filters were hybridized with ^{32}P-labeled subtracted cDNA probes generated essentially as described above. Positive clones were plaque purified and the cDNA inserts rescued from the phage by standard protocol. The cDNA inserts were subsequently purified for Northern blot analysis.

Genomic DNA Isolation and Southern Blot Analysis

Cells (1×10^7) were suspended in 5 ml of buffer containing 10 mM Tris-HCL, pH 7.5, 25 mM EDTA, 75 mM sodium chloride, and 200 μg/ml proteinase K and incubated at 37°C overnight. Subsequently, sodium chloride was added to a final concentration of 1.5 M and the solution was centrifuged at 3,500 rpm for 15 min to remove the precipitated protein. The DNA in the supernatant fluid was subsequently precipitated by the addition of 2.5 volume of cold ethanol. Genomic DNA was recovered with the aid of a glass rod, rinse in 70% of ethanol, and resuspended in 10 mM Tris-HCL, pH 8.0, 1 mM EDTA. The genomic DNA was digested with the specified restriction endonuclease and the digest was subsequently size-fractionated on a 0.7% agarose gel and transferred to nylon membrane overnight in 20 × SSC [Southern, 1975]. The DNA was immobilized by UV cross-linking as described above and the filter was hybridized and washed as previously described [Liau et al., 1985].

RESULTS

Isolation of Growth-Arrest Associated cDNAs

A cDNA library enriched in growth-arrest associated sequences was constructed using mRNA isolated from high density growth-arrested SMCs. Enrichment was accomplished by hybridizing the first strand cDNA with a 4-fold excess of mRNA isolated from SMCs stimulated with 20% FBS for 10 h. The nonhybridizing cDNA, isolated by hydroxylapatite chromatography, was used to construct the G_0 enriched cDNA library. The subtracted library was compared with two standard cDNA libraries constructed from high density growth-arrested SMCs by screening with GADD153, a known growth-arrest associated gene [Fornace et al., 1989]. The standard cDNA libraries contained one GADD153 positive clone per 5,000–7,500 plaques. By contrast, the subtracted cDNA library contained one positive clone per 750 plaques. We conclude that the subtracted cDNA library was roughly 10-fold enriched in growth-arrest associated DNA sequences.

Approximately 40,000 recombinant phages from the G_0-enriched cDNA library were subsequently screened with ^{32}P-labeled G_0 enriched cDNA or with a "background probe" comprised of ^{32}P-labeled G_0 cDNA subtracted with excess G_0 mRNA. Initially, 315 positive signals were detected with the G_0 enriched probe whereas only four enhanced signals were detected with the "background probe." Seventy-two of these positive clones were rescreened and 44 remained consistently positive. Nineteen of these positive clones were purified and the insert cDNA used for Northern blotting analysis to determine the expression of the corresponding mRNA in growth-arrested and serum stimulated SMCs. Although 11 of the 19 cDNAs encoded mRNAs that exhibited a higher level of expression in quiescent SMCs, most of the increase was only 2- to 3-fold (results not shown). However, as shown in Figure 1, one clone, designated LG7, hybridized to a 5.0 Kb mRNA that was induced approximately 10-fold in growth-arrested SMCs. This difference in LG7 mRNA level between SMCs stimulated with serum for 10 h and high density growth-arrest cells was comparable to that for the previously identified growth-arrest associated gene, GADD153.

Characterization of the Growth-Arrest Associated Gene LG7

We sequenced the 314 bp LG7 cDNA insert and found no significant identity with other sequences within the GenBank database. Using the G_0 enriched cDNA probe, we subsequently identified a second cDNA clone that also hybrid-

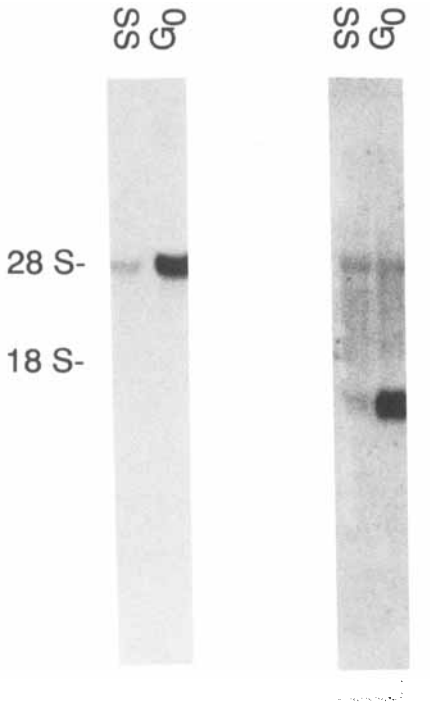

Fig. 1. Analysis of LG7 and GADD153 mRNA expression in proliferating and growth-arrested SMCs. RNA was prepared as described and the expression of LG7 and GADD153 was analyzed by Northern blot hybridization. SS = growth-arrested cells stimulated with 20% FBS for 10 h. G_0 = high density growth-arrested SMCs.

ized to a 5.0 Kb transcript that was induced approximately 10-fold in growth-arrested SMCs (results not shown). Since the mRNA size and growth-arrest associated induction of this second cDNA was very similar to that observed for LG7, it was possible that it also encoded the same gene. We sequenced the 402 bp cDNA insert of this second cDNA and found that the sequence did not overlap with that of the initial LG7 cDNA or with any other sequences in the GenBank database. We subsequently carried out genomic Southern blotting analysis with these cDNA inserts. Shown in Figure 2 is the genomic Southern pattern obtained from hybridizing with one of the cDNA fragments for LG7. The result was identical with the second cDNA fragment, indicating that LG7 encompassed both of the cDNA clones (results not shown). Interestingly, these cDNAs hybridized to multiple genomic DNA fragments, irrespective of the restriction enzyme. This was not due to incomplete digestion of the genomic DNA since unique bands were observed when a similar genomic blot was probed with a cDNA encoding another gene (Fig. 2). We next used the initial LG7 cDNA to screen a second cDNA library derived from high density growth-arrested SMCs, and cDNA clones that hybridized to both of the original cDNA fragments were purified and found to contain cDNA inserts of 1.2, 1.3, and 1.6 Kb. Southern blotting and partial DNA sequencing clearly indicated that these larger cDNAs were overlapping and encompassed both of the initial cDNA fragments for LG7 (results not shown). However, restriction mapping and additional DNA sequencing also revealed that certain regions were disparate between these cDNA clones. These results suggest that LG7 may be a member of a multigene family and/or that it may encompass a sequence found in other genes.

Enhance LG7 mRNA Expression Is Associated With Both High Cell Density and Low Serum Induced Growth-Arrest

We next characterized in greater detail the relationship between LG7 mRNA expression and cellular growth-arrest. To study LG7 mRNA expression during density-dependent growth inhibition, SMCs were plated out at a density of 3×10^3 cells/cm^2, and total RNA was isolated after 1, 2, 3, and 9 days. The cells were, respectively, 40, 60, and 80% confluent and 5 days postconfluent. The result, shown in Figure 3A, indicated that LG7 mRNA level increased with increased cell density and was maximum in 5 day postconfluent cells. Densitometry analysis of the Northern blot indicated there was a 7-fold increase in LG7 mRNA level between day 1 and day 9. By contrast, the level of actin mRNAs was unchanged in these cells and provided a control for RNA loading. We also examined the expression of LG7 in sparsely plated SMCs growth-arrested by serum starvation. Sparsely seeded SMCs were allowed to reach 50% confluence and switched to media containing 0.5% FBS, 10 μM insulin, and 5 μg/ml transferrin (low serum media). Total RNA was isolated from control cells and cells treated with low serum media after 24, 48, and 72 h for analysis of LG7 mRNA level. The result, shown in Figure 3B, indicated an increase in LG7 mRNA by 24 h. LG7 mRNA remain elevated at 48 and 72 h after serum withdrawal. By contrast the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was unchanged and served as a control. These results indicate that an increase in LG7 mRNA level is associated with both high density and low serum induced growth-arrest.

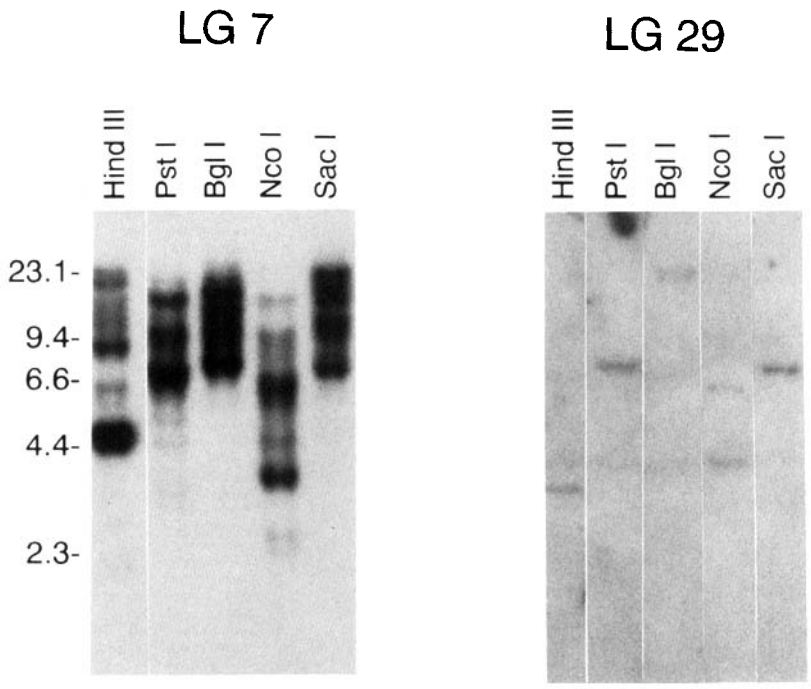

Fig. 2. Genomic Southern blot analysis of LG7. Genomic DNA was purified from cultured SMCs as indicated in experimental procedures and digested with the indicated restriction enzymes. Fifteen micrograms of DNA was used for each lane. The DNA was transferred to nylon filters and hybridized to ^{32}P-labeled LG7 and LG29 cDNAs.

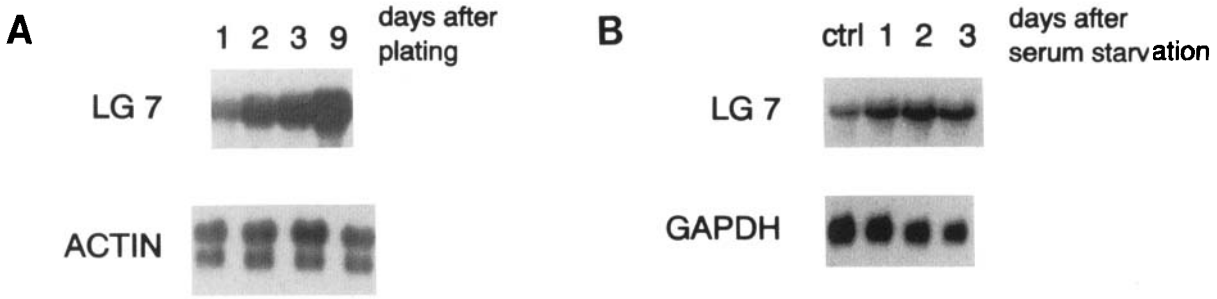

Fig. 3. LG7 mRNA expression in SMCs as a function of cellular density and serum deprivation. **A:** RNA was isolated from SMCs after various days in culture for analysis of LG7 mRNA level. Nine days in culture is equivalent to 5 days postconfluent. **B:** Growing cells were switched to low serum medium for the indicated number of days and subsequently harvested for RNA isolation. The actin and GAPDH probes were used as controls for total RNA loading.

Expression of LG7 Is Cell Cycle Modulated

Expression of other growth-arrest associated genes are known to be rapidly down-regulated as the cells traverse the cell cycle [Ciccarelli et al., 1990]. We next examined the mRNA expression of LG7 when confluent SMCs were growth-arrested by culturing in low serum containing media for 48–72 h and subsequently stimulated with FBS. The result, shown in Figure 4, indicated that by 4 h after the addition of serum there was a decrease in LG7 mRNA level. The level of LG7 mRNA remained low 24 h after serum addition. By contrast, the level of GAPDH was unchanged in these cells. To further investigate the cell cycle dependent expression of LG7 mRNA, we treated SMCs with compounds known to block cells at specific stages of the cell cycle. The result, shown in Figure 4, indicated that hydroxyurea, which arrests cells in early S-phase, caused a dramatic reduction in the mRNA level of LG7. By contrast, colchicine, which arrests cells in M-phase, increased the mRNA level of LG7 by 2–3-fold. The mRNA level of ferritin H-chain did not alter appreciably

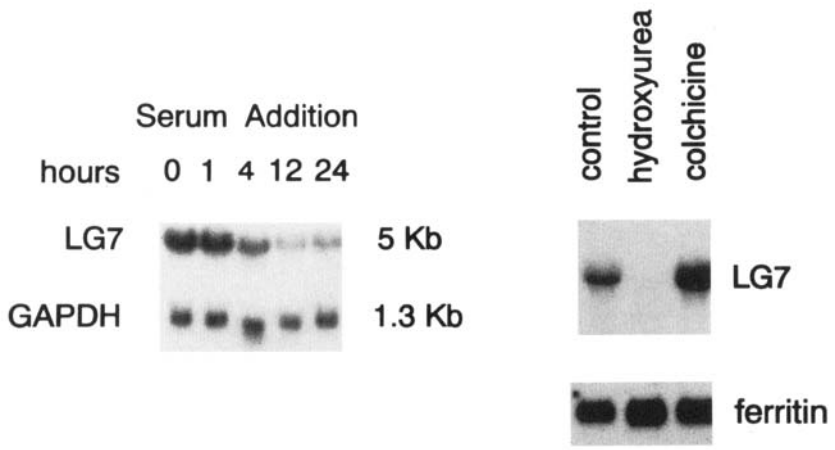

Fig. 4. LG7 mRNA expression in quiescent SMCs treated with 10% FBS and SMCs treated with cell cycle specific growth inhibitors. Cultured cells growth-arrested by incubation in low serum media were stimulated with 10% FBS for the indicated times and harvested for RNA isolation. Proliferating SMCs were also treated with 8 mM hydroxyurea or 8 μg/ml colchicine for 48 h. The GAPDH and ferritin H-chain probes were used as controls.

under these conditions. Additional studies with other cell cycle blockers revealed that aphidicolin (G_1-S block) and etoposide (G_2 block) also caused a reduction in LG7 mRNA level while Colcemid (mitotic block) caused an increase in LG7 mRNA level (results not shown). These results suggest that as cells enter S-phase there is a decrease in LG7 mRNA level, and its expression remains low during G_2 but returns to an elevated level during mitosis.

LG7 mRNA Expression Is Stimulated by cAMP and by Sodium Butyrate

We previously demonstrated that ferritin H-chain mRNA level was greatly enhanced in high density growth-arrested SMCs and that the addition of a cAMP analog to SMCs also caused an increase in ferritin H-chain mRNA expression [Liau et al., 1991]. It was, therefore, of interest to examine the possibility that cAMP may also stimulate an increase in LG7 mRNA level. SMCs were treated with 1 mM Bt_2cAMP and total RNA was isolated for various times and the expression of LG7 and ferritin H-chain was examined. The result, shown in Figure 5A, indicated that LG7 mRNA level increased significantly by 2 h after SMC exposure to Bt_2cAMP. The increase in LG7 mRNA level was maximal by 24 h and persisted for at least 48 h. By contrast, the level of ferritin H-chain mRNA did not increase significantly until 24 h after Bt_2cAMP addition. We also examined the effect of sodium butyrate, which suppresses SMC growth by a mechanism distinct from cAMP (Feng et al., manuscript submitted), on LG7 mRNA level. The result, shown in Figure 5B, indicated that treatment with sodium butyrate for 24 h alone slightly increased LG7 mRNA level but greatly enhanced the mRNA level of ferritin H-chain. Interestingly, when sodium butyrate was added in conjunction with the cAMP analog, 8Br-cAMP, there was a greater induction of LG7 then can be accounted for by either compound alone. This synergism was not evident with ferritin H-chain. The mRNA level of GAPDH was unchanged in these samples and represented an internal control.

Elevated LG7 mRNA Level Is Dependent on the Presence of a Protein With a Short Half-Life

Genes that are rapidly induced when quiescent cells are stimulated to proliferate have been well described, and a common characteristic of this class of genes is that they are superinduced in the presence of cycloheximide (CHX) [Lau and Nathans, 1991]. By contrast, expression of several of the growth-arrest specific genes identified in NIH 3T3s are down-regulated by CHX [Ciccarelli et al., 1990]. We examined the effect of CHX on LG7 mRNA expression and found that there was a decrease in LG7 mRNA levels by 8 h, and LG7 expression remained undetectable after 24 h (Fig. 6). By contrast, the expression of α_1(III) collagen and ferritin H-chain mRNA remained unchanged during this period (Fig. 6). Since CHX is known to have other

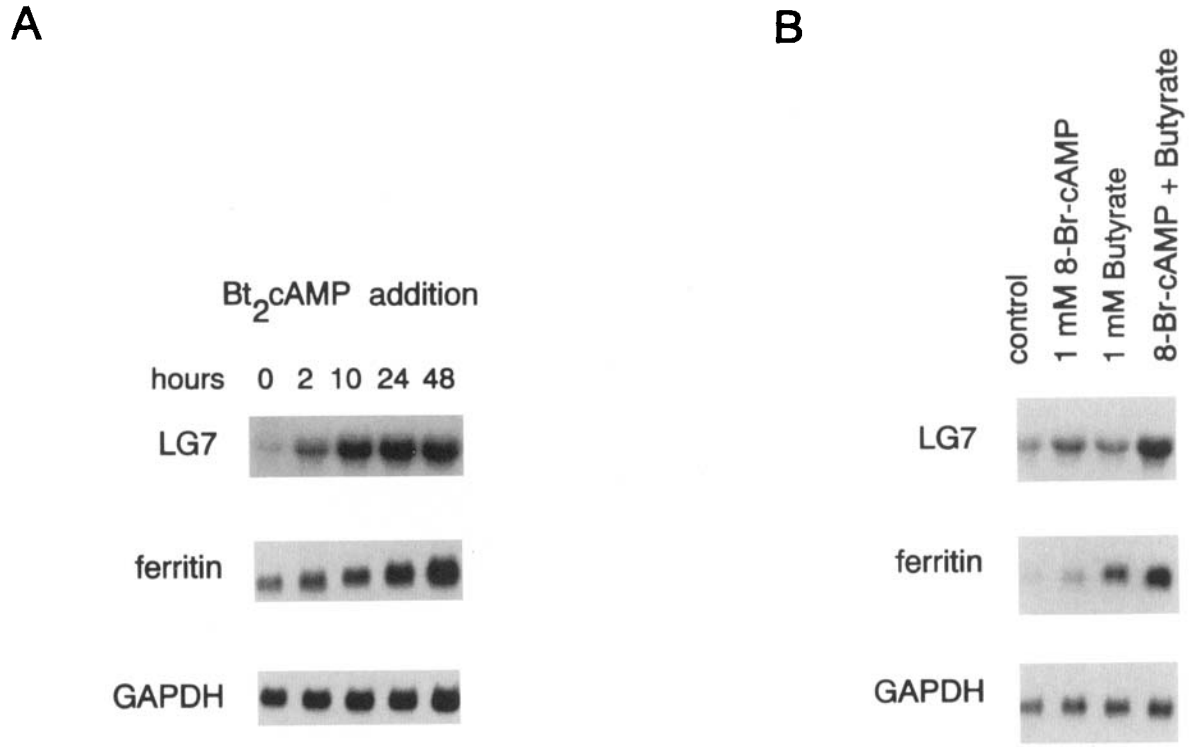

Fig. 5. Analysis of LG7 and ferritin H-chain mRNA level in response to the addition of cAMP analogs and to sodium butyrate. **A:** Cultured SMCs were incubated with Bt_2cAMP for the indicated times and harvested for RNA isolation. **B:** Growing cells were incubated with 1 mM 8-Bromo cAMP and 1 mM butyric acid or both for 24 h and harvested for RNA isolation. Northern blot analyses were carried out on these samples using the cDNA probes for LG7, ferritin H-chain, and GAPDH.

effects in addition to blocking protein synthesis, we also examined the effect of a second protein synthesis inhibitor, puromycin, on LG7 mRNA level. We found that puromycin inhibited LG7 mRNA level with similar kinetics as that for CHX, with a 6-fold decrease in LG7 mRNA level by 10 h after the addition of puromycin (results not shown). These results indicate that continued protein synthesis is necessary to maintain an elevated expression of LG7 mRNA.

DISCUSSION

We have used subtracted cDNA cloning to isolate cDNAs encoding a growth-arrest associated gene (LG7) in rabbit vascular SMCs. In addition, we found that GADD153/CHOP, a putative transcription factor believed to be involved in cellular growth-arrest [Barone et al., 1994], was also expressed at an elevated level in high density growth-arrested vascular SMCs. The relative increase in LG7 and GADD153 mRNA expression in high density growth-arrested SMCs vs. proliferating cells was similar. Based on mRNA size and preliminary sequencing data, LG7 is not one of the previously isolated growth-arrest associated genes [Schneider et al., 1988; Bedard et al., 1989; Fornace et al., 1989]. Interestingly, we found that various LG7 cDNAs contained internal regions with disparate sequences. One possible explanation for this finding is that LG7 mRNAs are alternatively spliced. A second possible explanation is that LG7 is one member of a multigene family. Additional data supporting the presence of a multigene family is that genomic Southern blotting analysis revealed the presence of multiple genomic DNA fragments that cross-hybridized to LG7. An alternative explanation for the presence of multiple genomic DNA fragments hybridizing to LG7 is that a sequence within the LG7 mRNA is also present in other unrelated genes. Since two nonoverlapping cDNA fragments gave identical patterns on genomic Southern blotting, this motif sequence must encompass both cDNA fragments which are approximately 800 bp apart. These results indicate that the genomic structure of LG7 is likely quite complicated and will require further characterization.

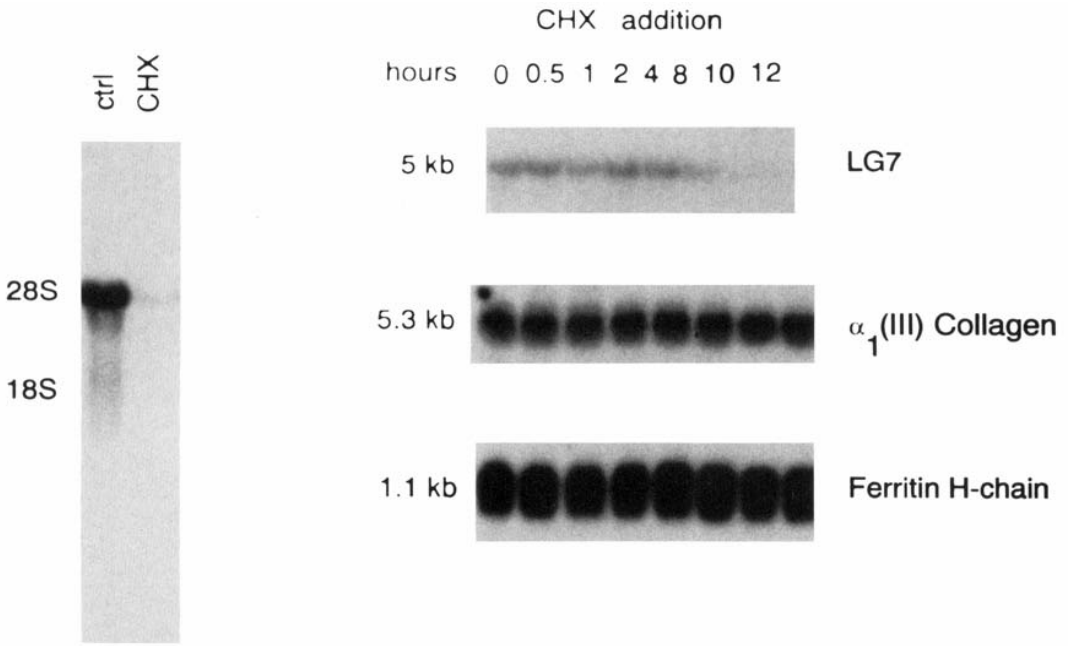

Fig. 6. The effect of the protein synthesis inhibitor cycloheximide on LG7 mRNA level in SMCs. Cells were treated with CHX (2 μg/ml) for the indicated times and RNA was collected for Northern blot analysis of LG7 mRNA level. Northern blot analysis of the same RNA samples were also carried out with the α_1(III) collagen and the ferritin H-chain DNA probes.

LG7 mRNA expression pattern is reminiscent of that reported for the growth-arrest specific (*gas*) genes [Schneider et al., 1988; Ciccarelli et al., 1990]. LG7 mRNA level increased during cell density as well as serum deprivation dependent growth-arrest, and addition of FBS caused a decrease in expression of LG7. Furthermore, by using cell cycle inhibitors that block progression at specific points, we found that LG7 mRNA levels were low when SMCs were blocked at G_1/S or at G_2 but LG7 mRNA was expressed at an elevated level when the cells were blocked at M-phase. However, our data suggests that factors other than cell growth are also important in the regulation of LG7 mRNA level. First, we have always been able to detect a basal level of LG7 mRNA in our cells irrespective of the growth state. Second, we have observed differences in the magnitude of LG7 mRNA decrease in response to serum. Finally, although both high cell density and serum deprivation are effective inhibitors of SMC growth in vitro, an increase in cell density is clearly a more effective inducer of LG7 mRNA expression. One possible explanation is the presence of a subpopulation of SMCs that is no longer capable of going through the cell cycle under certain conditions. Indeed, we find that SMCs allowed to achieve high cell density, subsequently exhibit poor growth rate and survival (Liau, unpublished observation).

The addition of cAMP analogs has been shown to inhibit the proliferation of vascular SMCs and to maintain contractile SMCs in a fully differentiated state [Chamley and Campbell, 1975; Jonzon et al., 1985; Southgate and Newby, 1990]. We previously determined that ferritin H-chain was expressed at an elevated level in high density growth-arrested rabbit SMCs and demonstrated that the addition of a cAMP analog caused an increase in the expression of this gene [Liau et al., 1991]. We report here that the cAMP induced increase in ferritin H-chain was a relatively slow process, suggesting that ferritin H-chain induction by cAMP is an indirect event, possibly due to the growth-inhibitory effect of this compound. Induction of GADD153 mRNA was also not apparent until 24 h after exposure to Bt_2cAMP (Liau, unpublished observation). By contrast, this cAMP analog induced an increase in LG7 mRNA level that was apparent by 2 h. This suggests that the increase in LG7 may

be directly regulated via the cyclic-AMP-dependent protein kinase pathway.

That LG7 mRNA was down-regulated in the presence of protein synthesis inhibitors is a particularly interesting finding since expression of the *gas* genes are also reduced by CHX addition to cells [Ciccarelli et al., 1990]. It is possible that down-regulation in the presence of protein synthesis inhibitors may be a common characteristic of this class of genes. By contrast, protein synthesis inhibitors appear to directly elevate the level of the serum-inducible immediate early genes and cause superinduction of these genes in the presence of serum [Lau and Nathans, 1991]. One interpretation of this result is that specific inhibitors of immediate early gene transcription are synthesized during early G_1. Similarly, it is possible that certain labile proteins need to be synthesized to maintain the elevated expression of the growth-arrest associated genes and that mitogenic stimulation specifically down-regulates the expression of these proteins. Candidates for these proteins include the transcription factor GADD153/CHOP and the homeobox gene Gax.

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