MOLECULAR AND CELLULAR BIOLOGY, June 1993, p. 3722–3733
0270-7306/93/063722-12$02.00/0

Vol. 13, No. 6

Molecular Cloning of a Diverged Homeobox Gene That Is Rapidly Down-Regulated during the G_0/G_1 Transition in Vascular Smooth Muscle Cells

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Received 7 January 1993/Returned for modification 15 February 1993/Accepted 10 March 1993

Adult vascular smooth muscle cells dedifferentiate and reenter the cell cycle in response to growth factor stimulation. Here we describe the molecular cloning from vascular smooth muscle, the structure, and the chromosomal location of a diverged homeobox gene, *Gax*, whose expression is largely confined to the cardiovascular tissues of the adult. In quiescent adult rat vascular smooth muscle cells, *Gax* mRNA levels are down-regulated as much as 15-fold within 2 h when these cells are induced to proliferate with platelet-derived growth factor (PDGF) or serum growth factors. This reduction in *Gax* mRNA is transient, with levels beginning to rise between 8 and 24 h after mitogen stimulation and returning to near normal by 24 to 48 h. The *Gax* down-regulation is dose dependent and can be correlated with the mitogen's ability to stimulate DNA synthesis. PDGF-AA, a weak mitogen for rat vascular smooth muscle cells, did not affect *Gax* transcript levels, while PDGF-AB and -BB, potent mitogens for these cells, were nearly as effective as fetal bovine serum. The removal of serum from growing cells induced *Gax* expression fivefold within 24 h. These data suggest that *Gax* is likely to have a regulatory function in the G_0-to-G_1 transition of the cell cycle in vascular smooth muscle cells.

Growth factor modulation of cell proliferation and differentiation is an important feature of normal development and has a role in many pathological conditions. This is especially true for vascular smooth muscle cells (VSMCs). Unlike adult cardiac and skeletal muscle cells, which are terminally differentiated and incapable of further cell division, vascular myocytes remain plastic and able to respond to growth factor signals released in response to endothelial injury by dedifferentiating and reentering the cell cycle (24, 40, 45). Upon entering the cell cycle, these cells activate certain genes, such as that for ornithine decarboxylase (11), and down-regulate others, such as that for smooth muscle-specific α-actin (8). In disease states such as atherosclerosis and coronary restenosis, regulation of vascular myocyte proliferation is disordered, resulting in the excessive growth of these cells and luminal narrowing and occlusion, which can ultimately compromise tissue perfusion (45). Determination of what nuclear factors might be involved in regulating vascular myocyte proliferation will thus be important to understanding the molecular basis of this regulation in VSMCs in normal and pathological states.

In G_0, growth and cell division are at a halt, but growth factor binding to receptors initiates a complex series of events causing cells to enter G_1, culminating with DNA replication during the S phase and ultimately cell division. Critical to this transition is the activation of several genes coding for transcription factors, which include the c-*fos*, c-*jun*, and c-*myc* proto-oncogenes, although their exact roles in modulating entry into the cell cycle are not well understood (58, 66). Potential negative regulators of this transition include the growth arrest-specific (*gas*) and growth arrest- and DNA damage-inducible (*gadd*) genes. These genes are expressed at high levels in quiescent NIH 3T3 fibroblasts or CHO cells, but their mRNA levels fall dramatically upon mitogen stimulation (9, 17, 18, 23, 28, 41, 55). Overexpression of one *gas* gene encoding an integral membrane protein (*gas1*) inhibits the entry of quiescent fibroblasts into the S phase, suggesting that it has a role in the maintenance of the quiescent state (23), although the mechanism by which this regulation occurs has not yet been determined.

Homeobox genes represent a class of transcription factors which, while long known to be important in cell differentiation and growth during embryogenesis, have yielded only hints that they might be involved in regulating the cell cycle. The proteins encoded by these genes are transcription factors with a helix-turn-helix motif that binds to AT-rich consensus sequences with high affinity (52, 57). Many, but not all, of these genes are located in one of four major HOX clusters and are expressed in the developing embryo in distinct overlapping spatial patterns along the anteroposterior axis which parallels their order along the chromosome (42). Homeobox transcription factors control axial patterning in the developing embryo, and they have also been implicated in the control of cell growth, differentiation, and tissue-specific gene expression. Examples of this regulation are found in the pituitary (GHF-1 or *pit-1*) (13) and the immune system (*oct-2*) (44), among others. Further evidence for the importance of these genes in growth regulation comes from the observation that some of them can, when overexpressed, be oncogenes (1, 62). In spite of this involvement in differentiation and growth in a wide variety of tissues, little is known about the regulation of homeobox genes by peptide growth factors, especially in mammalian cells. On the basis of the postulated roles of these genes in the control of organogenesis, lineage commitment, and cell growth, it is not unreasonable to expect that the expression of some homeobox genes could be regulated by extracellular growth

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factors or the cell cycle and might be involved in the transition from a quiescent to a proliferative state.

VSMCs provide a good model system to study cellular growth and differentiation processes because of their ability to take on a less differentiated morphology and reenter the cell cycle upon mitogen stimulation. Unlike skeletal muscle, where nuclear factors controlling myogenesis have been much more extensively studied (46, 67), little is known about the regulation of VSMC differentiation and proliferation at the nuclear level. Because of the widespread involvement of homeobox genes in developmental and growth control processes in other tissues, we decided to look for their presence in adult vascular smooth muscle in an effort to understand what nuclear factors might regulate its proliferation (48). Here we describe the isolation and characterization of a diverged homeodomain gene that is referred to as *Gax* (growth arrest-specific homeobox), to reflect the regulation of its expression in VSMCs. When quiescent VSMCs are stimulated by serum or platelet-derived growth factor (PDGF) to reenter the cell cycle, *Gax* expression is rapidly down-regulated with a time course similar to that of previously described *gas* genes (17, 18, 23), and the extent of this down-regulation is correlated to the mitogen's ability to stimulate ^{3}H-thymidine incorporation. Moreover, *Gax* expression is induced when proliferating cells are deprived of serum. These data suggest that *Gax* may have a regulatory role when quiescent VSMCs reenter the cell cycle.

MATERIALS AND METHODS

Hybridizations and cDNA cloning. An adult rat aorta cDNA library (36) in λ ZAP was screened with a 64-fold degenerate 29-mer oligonucleotide containing three inosine residues directed at the most highly conserved region of the *antennapedia* homeodomain (helix 3), with the following sequence (I = inosine): 5′-AA(A/G)ATITGGTT(T/C)CA(A/G)AA(C/T)(A/C)GI(A/C)GIATGAA-3′.

Recombinant phage colonies (500,000) in *Escherichia coli* were adsorbed in duplicate to nitrocellulose membranes and hybridized at 42°C with this oligonucleotide end labeled with [γ-^{32}P]ATP in a mixture containing 0.5 M sodium phosphate (pH 7.0), 7% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 1% bovine serum albumin (16). The filters were washed with a final stringency of 0.5× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0)–0.1% SDS at 42°C and exposed to X-ray film. Positive signals were isolated and rescreened until the clones were plaque purified. The plasmids containing the clones in λ ZAP vector were then excised by the protocol recommended by the manufacturer (Stratagene) and sequenced on both strands with Sequenase version 2.0 (United States Biochemicals). Homology searches were performed versus the GenBank and EMBL data bases (version 73) by using the BLAST algorithm (4).

Interspecific backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described elsewhere (19). A total of 205 F_2 mice were used to map the *Gax* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described previously (34). All blots were prepared with Zetabind nylon membranes (AMF-Cuno). The probe, a 1,155-bp rat cDNA clone, was labeled with [α-^{32}P]dCTP by using a random prime labeling kit (Amersham); washing was done with a final stringency of 0.2× SSCP (34)–0.1% SDS, 65°C. A major fragment of 4.2 kb was detected in *Hin*cII-digested C57BL/6J DNA, and major fragments of 3.6 and 2.7 kb were detected in *Hin*cII-digested *M. spretus* DNA. The 3.6- and 2.7-kb *M. spretus*-specific *Hin*cII fragments cosegregated and were monitored in backcross mice.

A description of the probes and restriction fragment length polymorphisms for the loci linked to *Gax*, including neuroblastoma *myc*-related oncogene 1 (*Nmyc-1*), the laminin B1 subunit gene (*Lamb-1*), a DNA segment, chromosome 12, the Nyu 1 gene (*D12Nyu1*), and the β-spectrin gene (*Spnb-1*), has been reported previously (5, 64). Recombination distances were calculated as described elsewhere (29) by using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Reagents and cell culture. Recombinant PDGF-AA, -AB, and -BB were obtained from Boehringer-Mannheim, and PDGF from human platelets was a gift from P. DiCorleto (Cleveland Clinic Foundation).

Cultures of rat aorta smooth muscle cells were obtained by enzymatic digestion of aortas isolated from adult male Sprague-Dawley rats according to previously described methods (39). Once established, the cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% bovine calf serum and were subcultured within 2 days after reaching confluence. Cells were stained with monoclonal antibodies to smooth muscle α-actin (Sigma Chemical Co.) to verify identity and were between passages 3 and 12 when used for experiments. Rat VSMCs immortalized with simian virus 40 large T antigen (a gift of C. Reilly, Department of Pharmacology, Merck Sharp & Dohme Research Laboratories) were cultured as described previously (51). NIH 3T3 fibroblasts, C2C12 myoblasts, and the rat embryonic aorta smooth muscle cell lines A7r5 and A10 were obtained from American Type Culture Collection and cultured as recommended by the supplier. Rat mesangial cells were a gift from M. Simonson (Department of Internal Medicine, Division of Nephrology, Case Western Reserve University) and were grown in RPMI medium with 17% fetal calf serum (FCS). For C_2C_{12} myoblasts, myotube formation was induced by incubating confluent cells for 3 to 4 days in Dulbecco's modified Eagle's medium with 2% horse serum, whereas myoblasts were harvested for RNA isolation at 50% confluence.

Mitogen inductions and ^{3}H-thymidine uptakes. For experiments in which rat aorta cells were stimulated with serum or PDGF, cells were plated at a density of 10 to 20% confluence, allowed to grow to >95% confluence (but not more dense than a monolayer), and then placed in low-serum medium containing 0.5% calf serum for 3 days. At this time, depending on the experiment, medium was removed from the cells and replaced with fresh medium containing FCS or PDGF. Cells were then incubated for the various times in the presence of mitogen and harvested for RNA isolation. As a control, quiescent cells were incubated with fresh serum-free medium alone. Experiments in which PDGF was added to medium without a medium change were also performed. In parallel, efficacy of mitogenic stimulation was confirmed by measuring VSMC ^{3}H-thymidine uptakes. Quiescent rat VSMCs at the same level of confluence as for the *Gax* down-regulation experiments were stimulated with mitogen and pulsed at various time points after stimulation for 1 h with 5 μCi of ^{3}H-thymidine per ml, after which trichloroacetic acid-precipitable counts were measured. In other experiments, sparsely plated cells growing in 20% FCS were

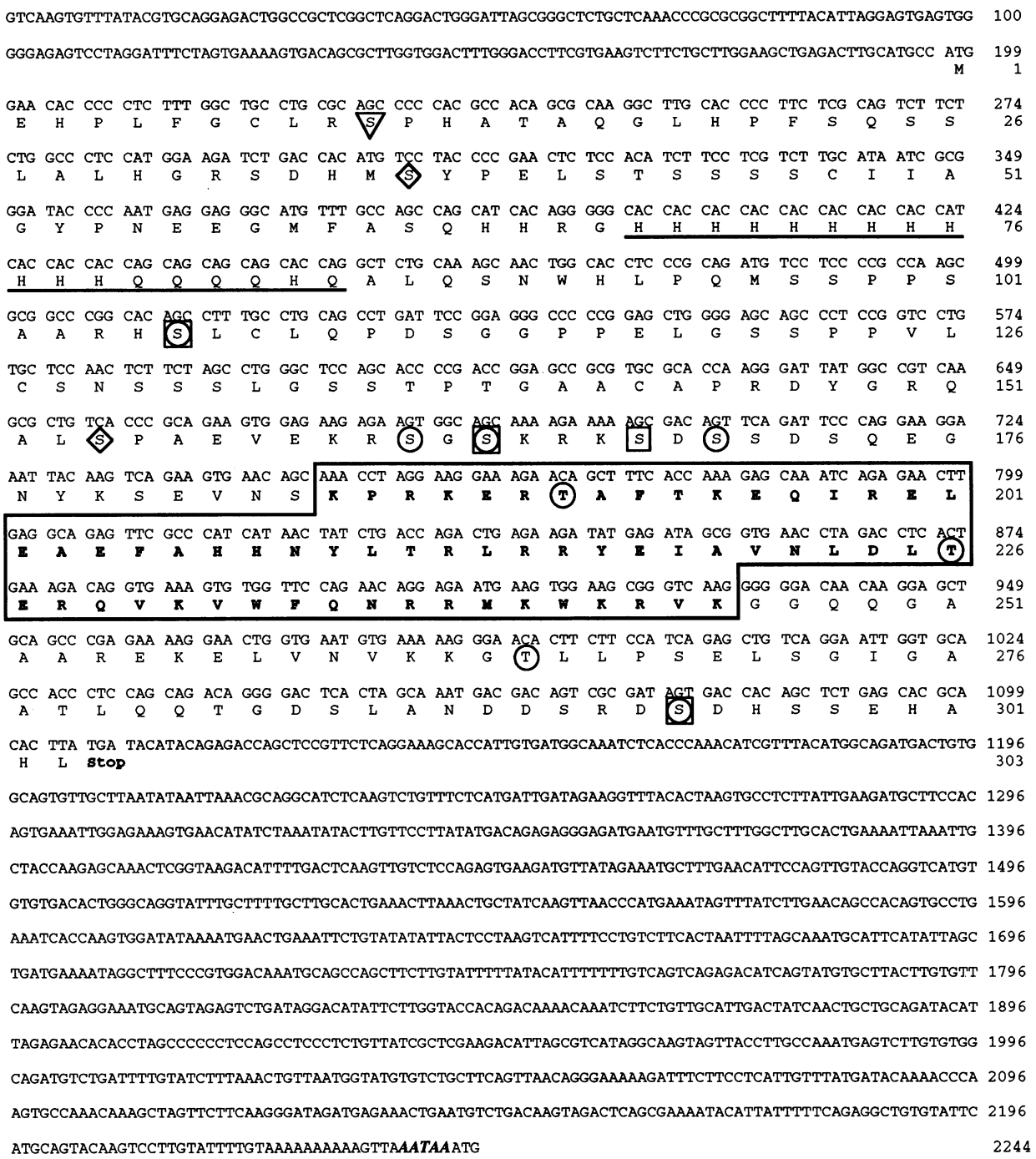


FIG. 1. Complete nucleotide and deduced amino acid sequences of *Gax*. The predicted amino acid sequence is indicated below the nucleic acid sequence. The homeobox is indicated by both boldface and a box, and the CAX repeat is underlined. A polyadenylation signal is in boldface and italics. Putative consensus sites are indicated as follows: for phosphorylation by protein kinase C, circles; for cyclic AMP (cAMP)-dependent protein kinase, squares; for casein kinase II, diamonds; and for histone H1 kinase, triangles. Residues which could potentially be a target for either cAMP-dependent protein kinase or protein kinase C are both circled and boxed. Consensus sequences for phosphorylation by protein kinases were obtained from Pearson and Kemp (49).

placed in serum-free medium and RNA was harvested at different time points for determination of *Gax* mRNA levels by Northern (RNA) blot analysis.

Northern blot analysis. Total RNA from rat tissue and cultured cells was prepared by the guanidine thiocyanate method (15), fractionated on 1.2% agarose gels containing formaldehyde, and blotted onto nylon membranes. RNA from organs and cultured cells (see Fig. 4A and B) was separated on 30-cm gels for transcript size determination and on 10-cm gels for other studies. Hybridizations were carried out at 65°C in the same buffer used to screen the library (16), using a cDNA probe labeled by random priming consisting of

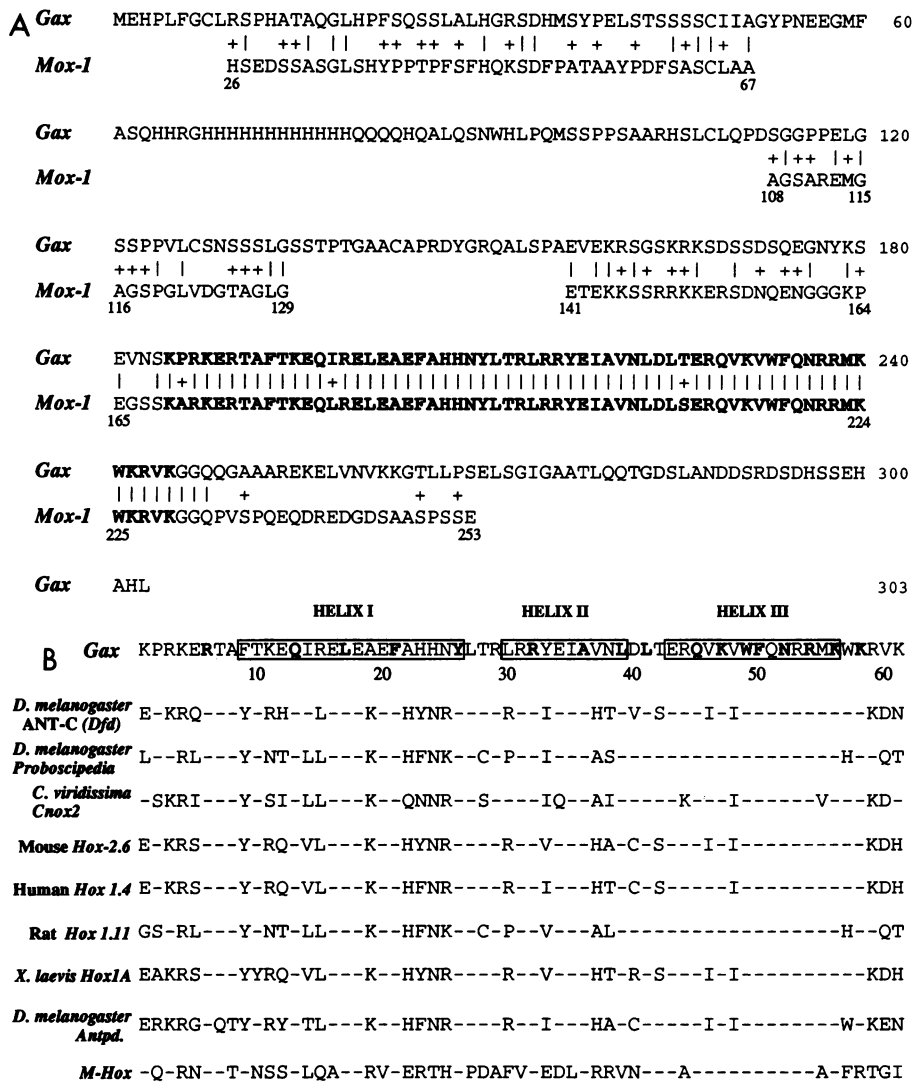


FIG. 2. *Gax* is homologous to *Mox-1* and more distantly related to the *Dfd* and *proboscipedia* homeobox genes. (A) Comparison of *Gax* with *Mox-1*. The homeodomains are indicated by boldface. Amino acid identities are indicated by lines, and conservative replacements are indicated by plus signs. The numbering of *Gax* amino acid residues is indicated to the right of the sequence, and the numbering of homologous *Mox-1* segments is indicated below the segments. The reported homeodomain of *Mox-2* is identical to that of *Gax* at the amino acid level. (B) Comparison of the *Gax* homeodomain with other homeodomains. Amino acid identities are indicated by dashes, and consensus residues for all homeodomains are indicated by boldface. The three homeodomain putative α-helices are indicated by boxes.

a truncated *Gax* cDNA lacking the 5′ end and the CAX repeat. Probes for *Hox-1.3* and *Hox-1.4* consisted of the cDNAs isolated from the rat aorta library, and the probe for *Hox-1.11* consisted of the *Dra*I-*Eco*RI fragment of its cDNA. Blots were washed with a final stringency of 0.1 to 0.2× SSC–0.1% SDS at 65°C. After all probings with the homeobox probes were complete, blots were rehybridized with a probe to rat glyceraldehyde 3-phosphate dehydrogenase to demonstrate message integrity.

For experiments involving the measurement of *Gax* down-regulation by mitogens, *Gax* and GAPDH mRNA levels for each lane were quantified with a Molecular Dynamics model 400S PhosphorImager to integrate band intensities. Alternatively, some experiments were quantified by scanning densitometry of autoradiograms. In all quantitative comparisons of *Gax* mRNA levels between experimental groups, *Gax* levels were normalized to the corresponding GAPDH level determined on the same blot, to account for differences in RNA loading.

Nucleotide sequence accession number. The nucleotide sequence of *Gax* has been submitted to the GenBank and EMBL data bases under accession number Z17223.

RESULTS

Primary structure of *Gax*. To identify homeodomain proteins present in vascular smooth muscle, an adult rat aorta cDNA library (36) was screened with degenerate oligonucleotides directed at the most highly conserved region of the homeobox, the third helix (see Materials and Methods). From 500,000 plaques, 13 positive clones were isolated, 12 of which contained homeodomains. Nine clones derived from previously described homeobox genes: *Hox-1.3* (26), *Hox-*

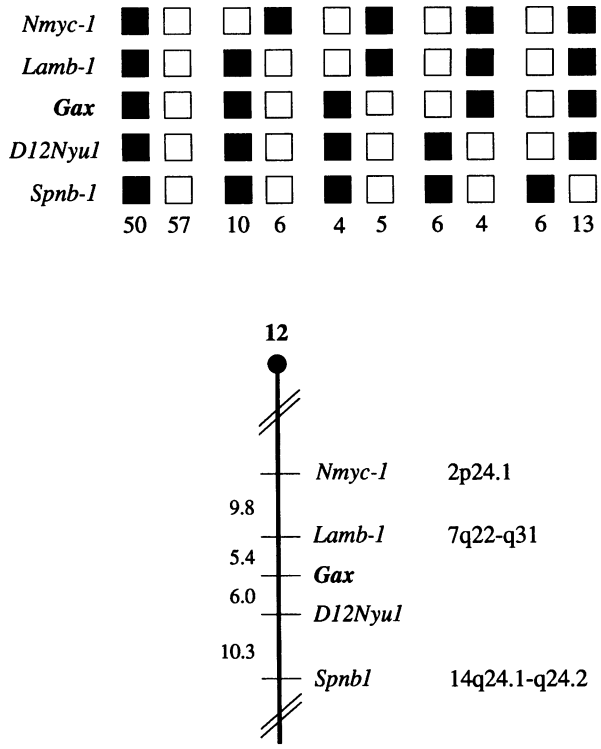


FIG. 3. *Gax* maps in the proximal region of mouse chromosome 12. *Gax* was placed on mouse chromosome 12 by interspecific backcross analysis. The segregation patterns of *Gax* and flanking genes in 161 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 161 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*) F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of an *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 12 linkage map showing the location of *Gax* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci mapped in this study can be obtained from Genome Data Base, a computerized data base of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, Md.).

1.4 (70), *Hox-1.11* (48, 65), and rat homeobox *R1b* (25). Three clones represented the cDNA designated *Gax*.

The cDNA encoding *Gax* is 2,244 bp in length, which corresponds to the size of the *Gax* transcript (2.3 to 2.4 kb) that is detected by Northern blot analysis (see below). The *Gax* cDNA has an open reading frame from nucleotide residues 197 to 1108 beginning with an in-frame methionine that conforms to the eucaryotic consensus sequence for the start of translation (37) and is preceded by multiple stop codons in all three reading frames. The open reading frame of the cDNA predicts a 33.6-kDa protein containing 303 amino acids with a homeodomain from amino acid residues 185 to 245 (Fig. 1). To confirm that this cDNA was capable of producing a protein product, the putative *Gax* open reading frame was fused in frame to the pQE-9 *E. coli* expression vector (Qiagen, Inc., Chatsworth, Calif.) and expressed in bacteria (33). *E. coli* containing this plasmid expressed a new protein of the predicted molecular weight, as determined by SDS-polyacrylamide gel electrophoresis, and extracts from these cells displayed a weak binding activity for the AT-rich, *MHox*-binding site (21) in the creatine kinase M enhancer (not shown).

The cDNA also contains a long 3′-untranslated region, from bases 1109 to 2244, with a polyadenylation signal at base 2237 (Fig. 1). The region between amino acids 87 and 184 is rich in serine (23 of 88 amino acids) and proline (10 of 88 amino acids) and contains several consensus sequences for phosphorylation by protein kinases (49). *Gax* also possesses a feature found in several transcription factors, including homeodomain proteins, known as the CAX or *opa* transcribed repeat (69). This repeat encodes a stretch of glutamines and histidines and in the case of *Gax* encodes 18 residues, of which 12 are consecutive histidines. This motif is shared by other transcription factors, such as the zinc finger gene *YY-1* (47, 59), as well as by several homeobox genes, including *H2.0*, *HB24*, *ERA-1* (*Hox-1.6*), *Dual bar*, and *Tes-1* (6, 22, 32, 38, 50). Although the function of the polyhistidine-polyglutamine domain encoded by the CAX repeat is unknown, we note that several of the aforementioned homeodomain genes are important in regulating the proliferation and differentiation of the cells in which they are normally expressed.

***Gax* is homologous to *Mox-1* and is distantly related to *Dfd*-class homeodomains.** Homology searches were performed versus GenBank with the peptide predicted by the *Gax* nucleotide sequence. *Gax* is relatively diverged from *antennapedia*, with only 54% amino acid identity (57). However, its homeodomain is nearly identical to that predicted for *Mox-1*, a recently described homeobox gene reported to be restricted to mesoderm and mesodermally derived tissues (12). There is a three-residue difference between the two predicted homeodomains at the amino acid level (95% identity), and all of these are conservative substitutions (Fig. 2A). At the nucleotide level, the homeodomains are 78% identical. Moreover, the 28-amino-acid sequence immediately upstream and the 3 amino acids immediately downstream from the *Gax* homeodomain are also homologous to the corresponding region of *Mox-1*. There are also two other regions upstream which share a lesser degree of homology. The *Gax* homeodomain is also identical at the amino acid level to the homeodomain recently reported for *Mox-2*, another mesodermal homeobox gene (12).

Gax is less homologous to members of recognized classes of homeodomains (57). It is similar to the *Drosophila* homeotic gene *proboscipedia* (20), which is identical to the *Gax* homeodomain in 40 of 61 amino acid residues (66% identity); *Hox-1.11*, with 39 of 61 amino acids being identical (64% identity) (65); and the recently reported *Chlorohydra viridissima* gene *Cnox2*, with 37 of 61 amino acids being identical (61% identity) (56). (Fig. 2B) The *Gax* homeodomain also shares homology with the homeodomains of several genes belonging to the class represented by the *Drosophila* gene *deformed* (*Dfd*), including the following: *Dfd*, 37 of 61 (61%) amino acids identical; *Hox-2.6*, 36 of 61 (59%) amino acids identical; *Xenopus laevis Hox1A*, 34 of 61 (56%) amino acids identical; and *Hox-1.4*, 37 of 61 (61%) amino acids identical (57) (Fig. 2B). However, despite the homology of its homeodomain with members of the *Dfd* class, *Gax* lacks other conserved elements characteristic of this class, such as the downstream pentapeptide LPNTK and a conserved N-terminal subfamily domain (2). Interestingly, the *Gax* homeodomain shares relatively little homology (26 of 61, or 43%,

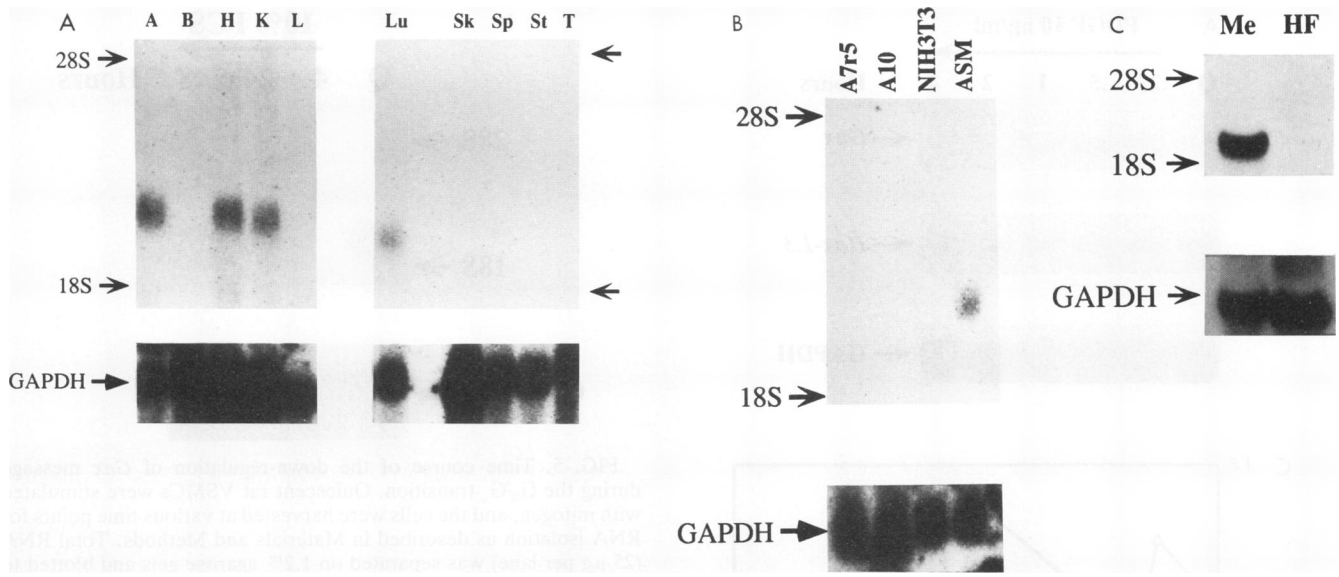
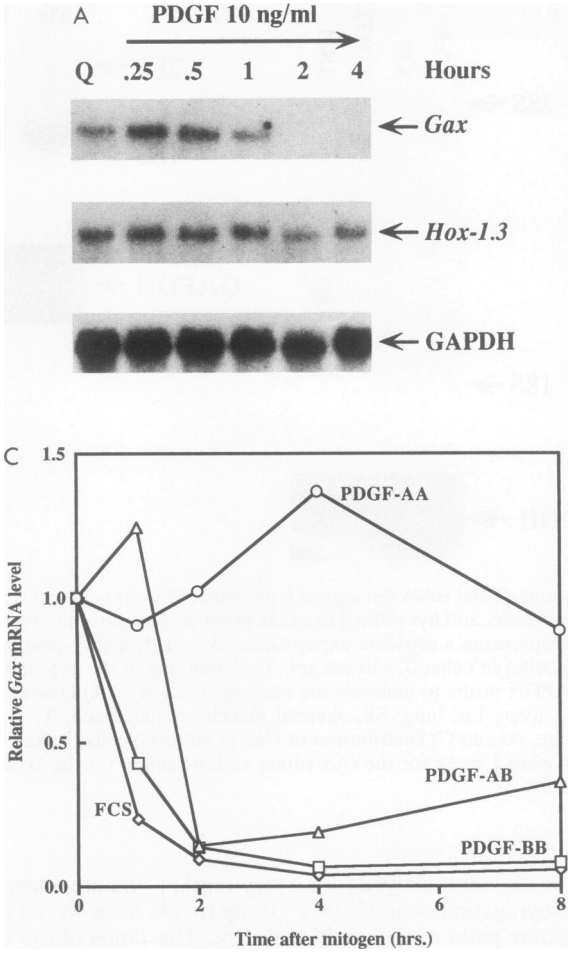


FIG. 4. Expression of *Gax* in adult rat tissues by Northern blot hybridizations. Total RNA (30 μg per lane) isolated from adult rat tissues was separated on agarose gels containing formaldehyde, blotted to a nylon membrane, and hybridized to a *Gax* probe as described in Materials and Methods, except for aorta, for which only 20 μg was used. Each panel represents a separate experiment. (A and B) Thirty-centimeter gels, done to determine the size of the *Gax* transcript in various tissues and cultured cells; (C) 10-cm gel. The positions of the 18S and 28S ribosomal bands are indicated by arrows. Blots were rehybridized with a GAPDH probe to demonstrate message integrity. (A) Distribution of *Gax* in adult tissues. Lanes: A, aorta; B, brain; H, heart; K, kidney; L, liver; Lu, lung; Sk, skeletal muscle; St, stomach; T, testes. Exposure times were 1 week for the *Gax* probe and 12 h for the GAPDH probe. (B and C) Distribution of *Gax* in cultured cells. Lanes: Me, mesangial cells; HF, human foreskin fibroblasts. In panel B, exposure times were 1 week for the *Gax* probe and overnight for the GAPDH probe; in panel C, exposure times were 4 days for *Gax* and 6 h for GAPDH.

amino acids identical) with that of *MHox*, the muscle-specific homeobox recently isolated by its ability to bind to an AT-rich site in the creatine kinase enhancer (21), whose human homolog (*phox*) has been shown to enhance the binding of the serum response factor to the serum response element (30). With such a lack of homology, it is unlikely that *Gax* shares any function with *MHox* or *phox*, despite the relative specificity of both gene products for certain types of muscle in the adult organism (see below). Because of the relative lack of homology between *Gax* and previously reported homeobox genes and the high degree of homology between *Gax* and *Mox-1*, these two genes likely constitute a new subfamily of homeobox genes, along with *Mox-2* (12). Given the high degree of sequence identity within their homeodomains, it is likely that members of this subfamily of homeodomain proteins recognize the same or very similar DNA-binding sites.

***Gax* maps to a locus on chromosome 12.** The mouse chromosomal location of *Gax* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 1,100 loci that are well distributed among all the autosomes as well as the X chromosome (19). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms with a rat cDNA *Gax* probe. The 3.6- and 2.7-kb *M. spretus Hin*cII restriction fragment length polymorphisms (see Materials and Methods) were used to monitor the segregation of the *Gax* locus in backcross mice. The mapping results indicated that *Gax* is located in the proximal region of mouse chromosome 12 linked to *Nmyc-1*, *Lamb-1*, *D12Nyu1*, and *Spnb-1*. Although 161 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 3), up to 193 mice were typed for some pairs of the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are as follows: centromere–*Nmyc-1*–19/193–*Lamb-1*–9/166–*Gax*–10/166–*D12Nyu1*–19/185–*Spnb-1*. The recombination frequencies (expressed as genetic distances in centimorgans ± the standard error) are as follows: *Nmyc-1*–9.8 ± 2.2–*Lamb-1*–5.4 ± 1.8–*Gax*–6.0 ± 1.9–*D12Nyu1*–10.3 ± 2.2–*Spnb-1*.

The mapping studies demonstrate that *Gax* is not a part of the HOX-1, HOX-2, HOX-3, and HOX-4 gene clusters, which are located on chromosomes 6, 11, 15, and 2, respectively (42), nor does it cosegregate with any other homeobox genes previously mapped in the interspecific backcross. *Mox-1* has been reported to reside on chromosome 11, and thus *Gax* and *Mox-1* do not form a novel homeobox gene cluster, although *Mox-2* has been mapped to chromosome 12 (12). On the basis of the high degree of homology between these genes, it can be speculated that they evolved through gene duplication, as has been proposed for members of the four known HOX clusters (57). Finally, we have compared our interspecific map of chromosome 12 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (compiled by M. R. Davisson, T. H. Roderick, A. L. Hillyard, and D. P. Doolittle and provided from GBASE, a computerized data base maintained at The Jackson Laboratory, Bar Harbor, Maine). *Gax* mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (data not shown).



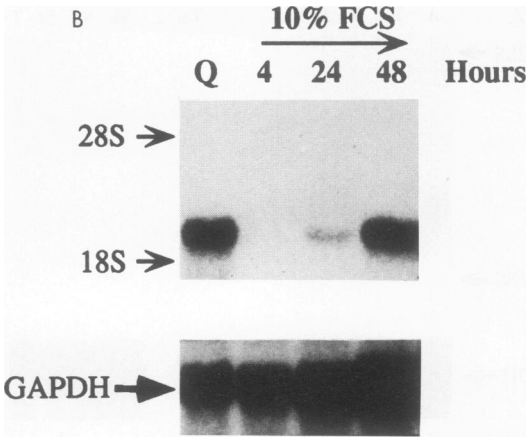


FIG. 5. Time course of the down-regulation of *Gax* message during the G_0/G_1 transition. Quiescent rat VSMCs were stimulated with mitogen, and the cells were harvested at various time points for RNA isolation as described in Materials and Methods. Total RNA (25 μg per lane) was separated on 1.2% agarose gels and blotted to nylon. (A) Short time course of *Gax* down-regulation by PDGF (10 ng/ml) isolated from human platelets. The same blot was serially reprobed with probes for *Hox-1.3* and GAPDH to demonstrate specificity of down-regulation to *Gax* and message integrity, respectively. (B) Longer time course experiment. Mitogen induction was performed as in panel A, except that 10% FCS was used as the mitogen, and cells were harvested for RNA isolation at later time points to demonstrate the slow recovery of *Gax* mRNA levels. The 48-h lane is slightly overloaded. Lanes Q, quiescent cells. (C) Quantitative measurements of *Gax* mRNA levels and the rate of down-regulation by the various mitogens as measured by the PhosphorImager (see Materials and Methods).

***Gax* expression in adult rat tissues and cultured cells.** The expression pattern of *Gax* was analyzed in adult rat tissues by Northern blot hybridization. Expression of the *Gax* transcript is largely confined to the cardiovascular system, including the descending thoracic aorta, where it is expressed at higher levels than in other tissues, and the heart (Fig. 4A). Expression was also detected in the adult lung and kidney. No expression was detected in the brain, liver, skeletal muscle, spleen, stomach, or testes (Fig. 4A), nor was expression detected in the intestine or pancreas (not shown). In contrast, *Gax* was more widely expressed in the developing embryo, with transcript detectable in the developing cardiovascular system, multiple mesodermal tissues, and some ectodermal tissues (not shown).

Gax expression was examined in cultured cells by Northern blot to determine its expression pattern and identify a cell type that could serve as a model to study *Gax* function. The 2.3- to 2.4-kb *Gax* transcript was detected in smooth muscle cells cultured from adult rat aorta (Fig. 4B), consistent with the in situ hybridization data (not shown) and the fact that *Gax* was originally isolated from a vascular smooth muscle library, and it was also detected in rat VSMCs transformed by simian virus 40 (not shown) (51). However, no expression was detected in either of two cell lines derived from embryonic rat aortic smooth muscle, A7r5 and A10 (Fig. 4B). *Gax* was also not detected in NIH 3T3 fibroblasts (Fig. 4B), human foreskin fibroblasts (Fig. 4C), or the skeletal muscle cell line C2C12, both myoblasts and myotubes (data not shown). A relatively high level of *Gax* expression was detected in cultured rat mesangial cells (Fig. 4C). Mesangial cells share many similarities to VSMCs, both structurally and functionally, and proliferate abnormally in renal diseases such as glomerulonephritis and glomerulosclerosis (43).

***Gax* is rapidly and transiently down-regulated during the transition from G_0 to G_1 induced by mitogen stimulation.** Because PDGF and other growth factors regulate vascular smooth muscle proliferation and differentiation, we assayed for differences in *Gax* expression that depended on the growth state of the vascular myocytes. Quiescence was induced in rat aorta VSMCs by serum deprivation for 3 days. A rapid down-regulation of *Gax* mRNA was observed when these quiescent VSMCs were stimulated with FCS or PDGF, potent mitogens for these cells (Fig. 5). The down-regulation ranged from 5- to nearly 20-fold, depending on the mitogen used and the experiment, typically occurred within 2 h after stimulation with serum or PDGF, and was maximal at 4 h. *Gax* transcript levels began to recover significantly by approximately 24 h and approached baseline between 24 and 48 h after stimulation. This rate of recovery tended to vary with the magnitude of the initial down-regulation and depending on the individual rat VSMC preparations. A typical experiment is shown in Fig. 5A, using PDGF isolated from human platelets as the mitogen, and a longer time course experiment is shown in Fig. 5B, using FCS as the mitogenic stimulus. Rat VSMCs immortalized with the simian virus 40 large T antigen retain many characteristics of normal VSMCs, including growth inhibition by heparin (51), and

also down-regulate *Gax* in response to FCS in a manner identical to that of normal rat VSMCs (data not shown). Pretreatment of the either normal cultured rat VSMCs or transformed rat VSMCs with cycloheximide attenuated the down-regulation at 4 h by as much as 70% (data not shown), suggesting a requirement for new protein synthesis as a major component of the down-regulation. Furthermore, treatment of quiescent and PDGF- or serum-stimulated cells with actinomycin D demonstrated no gross change in mRNA stability (not shown), suggesting that the mechanism of the down-regulation may be transcriptional.

PDGF is a homo- or heterodimer made of any combination of two chains, A and B, linked by disulfide bonds. Thus, there are three isoforms of PDGF—PDGF-AA, PDGF-AB, and PDGF-BB (53)—and they have differing potencies for stimulating DNA synthesis in rat VSMCs (54). PDGF-AA is a relatively weak mitogen in VSMCs, whereas and PDGF-AB and -BB are much more effective, with the BB isoform being slightly more potent. PDGF-AA at 10 ng/ml did not down-regulate *Gax* expression in quiescent VSMCs, whereas the PDGF-AB or -BB isoform (both at 10 ng/ml) or 10% FCS reduced *Gax* expression approximately 10-fold by 4 h (Fig. 5C). Qualitatively the effects were the same with these mitogens, although quantitatively the magnitude of the down-regulation was greatest with the FCS followed by that with PDGF-BB and PDGF-AB. The mitogen-induced down-regulation was specific for *Gax* and not a general feature of homeobox genes expressed in VSMCs. While PDGF isolated from human platelets caused a rapid down-regulation of *Gax*, it had little or no effect on *Hox-1.3* mRNA levels (Fig. 5A). Neither serum nor any of the three isoforms of PDGF detectably affected the transcript levels of *Hox-1.3*, *Hox-1.4*, or *Hox-1.11*, homeobox genes which were also isolated from the vascular smooth muscle library (not shown).

We wished to determine whether the extent of *Gax* down-regulation correlated with the potency of the mitogen used to stimulate the VSMCs. Therefore, we compared the three PDGF isoforms and FCS in their mitogenic potencies, as measured by their ability to stimulate ^{3}H-thymidine uptake (Fig. 6). PDGF-AA at 10 ng/ml, which was completely ineffective in causing *Gax* down-regulation (Fig. 5C), stimulated DNA synthesis only weakly (Fig. 6 and data not shown). The PDGF-AB and -BB isoforms both stimulated cell proliferation as measured by ^{3}H-thymidine uptakes at 15 h, but FCS was the most effective mitogen. Dose-response experiments revealed that the 50% effective dose for *Gax* down-regulation 4 h after mitogen stimulation is between 4 and 8 ng/ml and 2 and 5 ng/ml for PDGF-AB and -BB, respectively (Fig. 7A). Furthermore, a full mitogenic dose of 10% FCS suppresses *Gax* levels nearly 20-fold at 4 h (Fig. 7B), an effect larger than that of a maximal stimulatory dose of PDGF-BB (30 ng/ml), which has a 10-fold effect, or of PDGF-AB, which has a less than 8-fold effect (Fig. 7A). Thus, the down-regulation of *Gax* induced by either serum or the different isoforms of PDGF correlates well with their relative abilities to stimulate ^{3}H-thymidine uptake by these cells.

Another feature of this down-regulation is that it is sensitive to low levels of mitogen stimulation, which can cause a significant decrease in *Gax* mRNA levels. For instance, stimulation of quiescent rat VSMCs with 1% FCS caused a 40% decrease in *Gax* mRNA levels after 4 h (Fig. 7B), even though such stimulation increased ^{3}H-thymidine uptake less than twofold over that observed in quiescent VSMCs (data not shown). Treatment with PDGF-BB at doses as low as 2 ng/ml also caused a detectable decrease in the *Gax* message level. This concentration is well within the physiological range of this mitogen, and thus it seems likely that *Gax* down-regulation also occurs in vivo, perhaps when VSMCs are induced to proliferate by blood vessel injury and subsequent growth factor release.

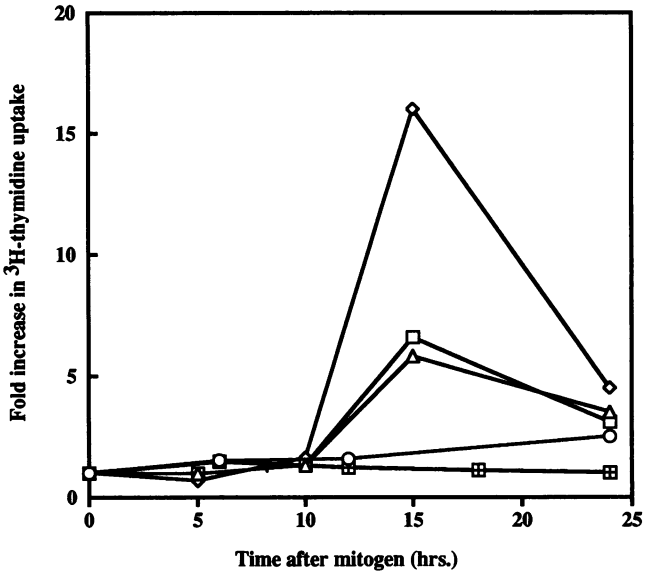
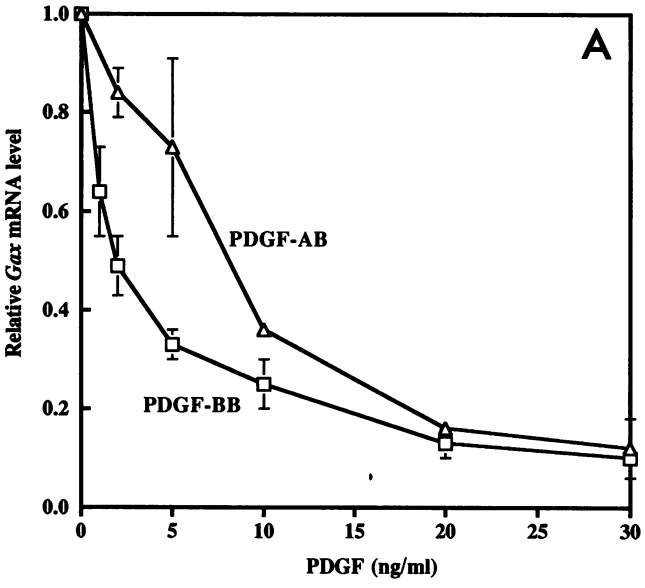


FIG. 6. Time course of VSMC entry into S phase as measured by ^{3}H-thymidine uptake after mitogen stimulation. Quiescent cells were stimulated with mitogen and pulsed with ^{3}H-thymidine for 1 h at various time points, and acid-precipitable counts were measured as described in Materials and Methods. Each value for ^{3}H-thymidine uptake represents the mean from four wells. Circles, PDGF-AA; triangles, PDGF-AB; squares, PDGF-BB; diamonds, FCS; squares with cross, no mitogen.

Finally, in terms of its time course, the down-regulation of *Gax* significantly precedes the cells' entry into S phase and the activation of c-*myb*, which occur 12 to 16 h and 8 to 10 h after mitogen stimulation, respectively (10, 54). The recovery of *Gax* message levels appears to begin sometime in late S phase or in G_2, as *Gax* is detectable at 24 h (Fig. 5B). *Gax* message levels return to nearly normal approximately 48 h after the initial stimulation, perhaps because of density-dependent growth inhibition. Similar behavior has been noted for *gas1* and *gas5*, whose transcripts begin to reappear approximately 10 to 18 h after serum stimulation (17, 55). Finally, the expression of *Gax* is induced fivefold in VSMCs within 24 h after rapidly growing, sparsely plated cells are placed in serum-free medium (Fig. 8). This time course is similar to that of the induction of *gas* genes in fibroblasts in response to serum starvation (17, 41, 55). Thus, expression of *Gax* is regulated by the growth state of the cell, and its down-regulation is a prominent feature of the G_0/G_1 transition in these cells.

DISCUSSION

It is now well established that homeodomain genes are critical in many processes during development involving pattern formation, lineage commitment, organogenesis, and regulation of cell differentiation and proliferation (42, 52, 57). Evidence has also been accumulating that the deregulation of homeobox gene expression can lead to uncontrolled cell



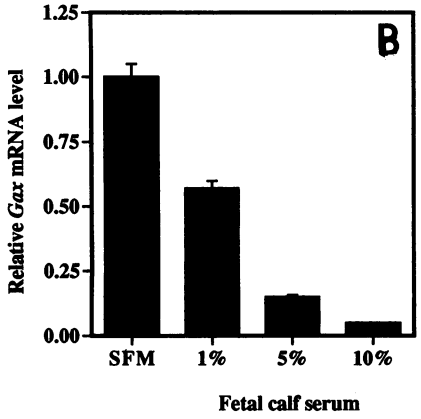


FIG. 7. Dose-response curves for PDGF and serum in causing the down-regulation of *Gax*. Quiescent VSMCs were stimulated with either PDGF-AB, PDGF-BB, or FCS at differing doses, and the effect on *Gax* mRNA levels was measured at 4 h after mitogen stimulation, as described in Materials and Methods. (A) Dose-response curve for PDGF-AB and -BB; (B) dose-response curve for FCS. Curves represent the means from two to three experiments. SFM, serum-free medium.

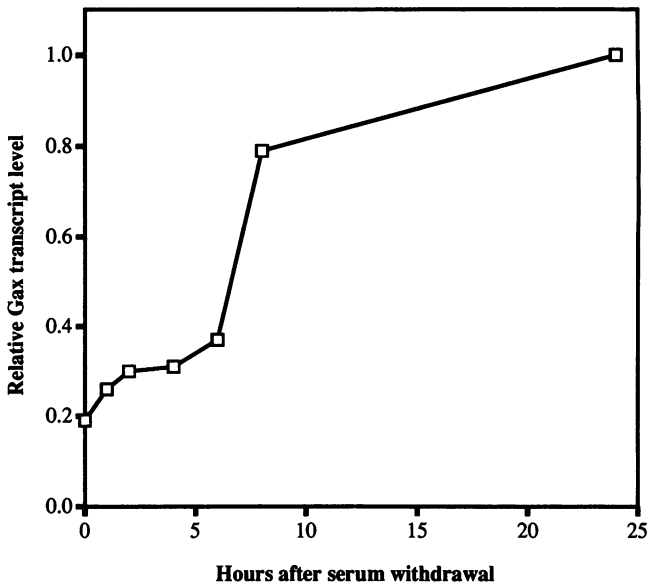


FIG. 8. Induction of *Gax* expression in response to serum deprivation. Sparsely plated VSMCs growing in medium containing 20% FCS were placed in serum-free medium, and RNA was harvested at various time points for Northern blot analysis and subsequent quantitation of *Gax* mRNA levels as described in Materials and Methods. Values for *Gax* message level were normalized to the intensity of the GAPDH signal.

growth and disease (1, 31, 62). In this study, we have isolated a divergent homeobox cDNA, *Gax*, from a vascular smooth muscle library, and this gene maps to mouse chromosome 12 by interspecific backcross analyses. In the adult rat, its transcript can be detected only in the aorta, heart, kidney, and lung. The localization of *Gax* transcripts in vascular muscle is of interest because these cells have a key role in coronary restenosis and the development of atherosclerotic lesions. Evidence for *Gax* expression in vascular myocytes includes the isolation of this cDNA from an adult aorta library, the detection of *Gax* transcripts in adult aorta by Northern blot (Fig. 4A) and in situ hybridization (not shown), and the detection of *Gax* expression by Northern blot in vascular myocytes that were cultured from adult rat aorta (Fig. 4B) and in simian virus 40-transformed smooth muscle cells (not shown). The *Gax* mRNA is also detected in whole kidney and in mesangial cells cultured from this organ (Fig. 4C). Mesangial cells share many similarities to VSMCs, both phenotypically and in their ability to respond to growth factors such as PDGF, and their abnormal proliferation is important in the pathogenesis of various forms of glomerulosclerosis and glomerulonephritis (43).

Vascular myocytes differ from skeletal and cardiac myocytes in that they are capable of reentering the cell cycle in response to mitogen stimulation, a critical feature of many blood vessel diseases. A unique feature of *Gax* is that it is expressed in quiescent VSMCs, but it is down-regulated from 5- to greater than 15-fold when these cells are induced to proliferate by mitogens. The down-regulation occurs rapidly during the transition from G_0 to G_1, and it is transient, with *Gax* levels recovering much later in the cell cycle. Furthermore, the magnitude of the *Gax* down-regulation correlates with the mitogenic potential of the growth factor. The most potent mitogenic stimulus examined, FCS, produced the largest down-regulation, and the recovery of *Gax* to prestimulation levels was slowest with this mitogen. Stimulation with physiological concentrations of PDGF-AB and -BB also led to the down-regulation of *Gax*, with the BB isoform being slightly more potent. However, PDGF-AA, a weak mitogen for VSMCs, had no effect on *Gax* levels and was also ineffective at stimulating ^{3}H-thymidine uptake. It has been shown that PDGF-AA activates intracellular signaling pathways different from those activated by the AB and BB isoforms (54), which may explain its inability to down-regulate *Gax*.

In quiescent VSMCs, growth factors induce the expression of the nuclear proto-oncogenes c-*fos*, c-*myc*, and c-*myb* (10, 35), and these cells enter the S phase 10 to 16 h after stimulation (54) (Fig. 6). Of particular interest, c-*myb*, which is activated in late G_1 and S phase, serves as an effective antisense oligonucleotide target to prevent VSMC proliferation in an in vivo rat model of coronary restenosis (61). Since the down-regulation of *Gax* significantly precedes the up-

regulation of c-*myb* (10), the induction of DNA synthesis (54), and the down-regulation of smooth muscle contractile proteins (7), it is possible that the reduction of *Gax* expression is in some way involved in the mitogenic effect of serum or PDGF on VSMCs. This possibility seems even more likely, given that the other homeobox genes isolated from these cells (*Hox-1.3*, *Hox-1.4*, and *Hox-1.11*) are insensitive to mitogen stimulation, making *Gax* unique among the homeobox genes expressed in VSMCs.

Despite the importance of homeobox genes in the control of cell fate and proliferation, only recently has attention focused on the modulation of these genes by peptide growth factors and other mitogens. Specifically, basic fibroblast growth factor and members of the transforming growth factor β family have been shown to regulate homeobox gene expression in developing *Xenopus* embryos (3, 14). Less, however, is known about regulation of homeobox gene expression by peptide growth factors in mammals, although cytokines have been shown to be capable of activating homeobox gene expression in the human immune system (22). Thus, it is clear that growth and differentiation factors can be regulators of homeobox gene expression. We note, however, that the regulation of *Gax* differs from that of these other homeobox genes in that it is down-regulated by mitogens rather than up-regulated and the time course of the effect is much more rapid. Whereas *Gax* down-regulation is significant by 2 h and maximal by 4 h, the up-regulation of other homeobox genes by basic fibroblast growth factor or interleukin-2 takes many more hours (3, 14, 22) and in the case of the differentiation agent retinoic acid can take days (60). The rapid time course of *Gax* down-regulation suggests that it may have a more direct role in cell cycle regulation than these other homeobox genes.

Evidence that homeobox genes are indeed directly involved in growth control in many cell types is accumulating. There are now examples of homeobox genes that can act as oncogenes. For instance, transfection of activated *Hox-2.4*, in which the gene's promoter has a mutation causing constitutive expression, into NIH 3T3 cells yields cells capable of forming fibrosarcomas in nude mice (1), and overexpression of *Hox-7.1* in myoblasts inhibits terminal differentiation and causes cell transformation (62). Little, however, is known about the potential involvement of homeobox genes in regulating the cell cycle, although there are tantalizing hints. For example, *oct-1* has been implicated in the cell cycle-specific expression of histone H2B (27). Evidence of the ability of homeobox genes to regulate the activity of immediate-early gene products also comes from the recently described human gene *phox* (whose mouse homolog is *MHox*), which has been shown to enhance the binding of bacterially produced serum response factor to the serum response element in the c-*fos* promoter (30). Whether *Gax* interacts with immediate-early genes remains to be proved; however, given its rapid down-regulation when quiescent cells reenter the cell cycle, it is possible that this gene is involved in the control of proliferation in VSMCs, either by repressing genes necessary for the G_0/G_1 transition or by activating tissue-specific genes or other genes associated with the quiescent state.

The rapid down-regulation of *Gax* in VSMCs in response to mitogens and its up-regulation when cells exit the cell cycle follow time courses that strongly resemble those observed with *gas* genes in NIH 3T3 fibroblasts (17, 18, 23, 41, 55). Dissecting the function of previously isolated *gas* genes at the molecular level may be difficult because the proteins they encode either show little homology to known proteins or belong to classes of proteins whose detailed molecular functions are difficult to study. At least two *gas* genes (*gas1* and *gas3*) encode integral membrane proteins (23, 41, 63, 68), and one (*gas2*) encodes a protein which has been shown to be a component of the microfilament system (9). A transcription factor such as *Gax* is likely to be more amenable to detailed molecular dissection of its function, such as the determination of its DNA consensus binding site and mapping of activation and repression domains, both of which *Gax* possesses (30a). Future studies will test whether *Gax* has a direct growth repression function like *gas1* (23) or whether *Gax* functions by responding to proliferative signals and thereby transmits this information to downstream genes. Determination of the downstream targets of *Gax* will be of great interest, as this information could potentially yield new insights into the mechanisms by which homeobox genes coordinate cell differentiation and proliferation.

ACKNOWLEDGMENTS

We thank D. J. Gilbert for excellent technical assistance.

This research was supported by National Institutes of Health grants AR40197 and HL45345 (to K.W.), NEO Affiliate American Heart Association fellowship F286 (to C.V.P.), and by the National Cancer Institute, DHHS, under contract NO1-CO-74101 with ABL. D.H.G. is an Allen Fellow in the Department of Surgery, University Hospitals of Cleveland, and is also partially supported by the Cleveland Cardiovascular Training Grant. K.W. is an established investigator of the American Heart Association.

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