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Homeobox gene expression and regulation in vascular myocytes

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Case Western Reserve University (Health Sciences), 1994

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HOMEOBAX GENE EXPRESSION AND
REGULATION IN VASCULAR MYOCYTES

by

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Submitted in partial fulfillment of the
requirements for the degree of Doctor of
Philosophy

Thesis Advisor: Dr. Kenneth Walsh

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HOMEBOX GENE EXPRESSION AND REGULATION
IN VASCULAR MYOCYTES

Abstract

by

DAVID HENRY GORSKI

This dissertation examines the potential roles of homeobox genes in growth regulation in vascular smooth muscle cells, a topic whose interest derives from its relevance to disease processes in which vascular myocyte proliferation is disordered, such as atherosclerosis. Abnormal proliferation of vascular smooth muscle cells is a critical component of the pathogenesis of both of these conditions, and very little is known about transcription factors which might play a role in regulating the entry of these cells into the cell cycle. To determine if homeobox transcription factors are involved in this process, a vascular cDNA library was screened with a degenerate oligonucleotide probe directed at the most conserved region of the homeodomain. Several homeobox cDNAs were isolated, including one that encodes Gax, a diverged homeobox gene that was subsequently found to be rapidly down-regulated when quiescent vascular smooth muscle cells were stimulated with serum growth factors. Further studies revealed another, previously described, homeobox gene which is responsive to serum growth factors, MHOX/phox, whose transcript is rapidly up-regulated upon mitogen stimulation. Because of its unique pattern of expression, we tested recombinant Gax protein for its ability to inhibit the entry of vascular smooth
muscle cells into S-phase. Gax inhibited the entry of quiescent vascular myocytes into S-phase by 40%. We compared this to MyoD and an anti-ras antibody, which inhibited vascular myocyte entry into S-phase by 36% and 65%, respectively. The growth inhibitory effect of microinjected Gax was dose-dependent and reversed by highly oncogenic ras mutant, Ras[Leu-61]. Time course microinjection experiments suggested that Gax acts somewhere in late G1 to inhibit vascular myocyte proliferation. These experiments suggest that Gax is a negative regulator of growth in vascular myocytes, and that it is likely that Gax and other homeobox genes play a role in regulating the modulation of vascular myocytes from the contractile to the synthetic phenotype.
DEDICATION

To Joanne, who put up with me as I wrote this.
ACKNOWLEDGMENTS

I wish to express my thanks to Dr. Walsh, for his guidance over the last three years; to Dr. Shuck, for supporting me through this longer-than-expected process; to Chandrashekhar Patel, for his help and practical advice during this entire project; and to all the members of the Walsh laboratory with whom I've worked, past and present, all of whom contributed to this project and to my education.
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CHAPTER 1:

HOMEOBOX GENES AND VASCULAR SMOOTH MUSCLE CELLS

Introduction

Remodeling in the cardiovascular system, both at the cellular and organ level, occurs during normal development and in various pathological states. Examples during embryogenesis include the fusing of the paired heart tubes to form the primordial heart, the partitioning of the heart to form the atria and ventricles, and the formation of the vascular system from the aortic arches. Postnatal examples include changes in the cardiovascular system necessary for the lungs to function in oxygen exchange. These include the closure of the ductus arteriosus, ductus venosus, and foramen ovale, and the hypertrophy of the left ventricle, which allows it to handle the increased work load associated with the systemic circulation [1]. Two examples of cardiovascular disease states where cellular remodeling plays a prominent role in their pathophysiology include cardiac hypertrophy and atherosclerosis [2-14]. Indeed, these processes appear to involve a return of the cell or tissue to a less differentiated state, more like that found in the embryo [3-6, 8, 9, 12, 13]. For instance, vascular smooth muscle cells (VSMCs), when stimulated by mitogens, migrate to the intima of the vessel, downregulate the expression of contractile proteins, and proliferate [4, 15], and cardiac myocytes undergoing hypertrophy express skeletal muscle actin isoforms normally expressed in the fetal heart only during development [2, 14]. Moreover, the ratio of nonmuscle to smooth muscle-specific isoforms of actin and myosin expressed in
smooth muscle cells isolated from atherosclerotic plaques resembles that found in vascular smooth muscle during early development. Nonmuscle isoforms predominate, and these cells proliferate at increased rates when placed in tissue culture [4, 5, 15-17]. In contrast to skeletal muscle [18-23], little is known about the detailed molecular biology of transcription factors which regulate tissue-specific gene expression and control the growth and differentiation of vascular smooth muscle and cardiac muscle.

Homeobox transcription factors are known to be regulators of pattern formation, cell differentiation, proliferation, and migration in vertebrates and invertebrates [24-57]. These properties make them promising candidates for being regulators of VSMC growth and differentiation. Recently, several of these factors have been isolated from or detected in the tissues of the cardiovascular system [35, 36, 53]. Because of their role in these critical processes, our laboratory's interest in transcription factors and muscle differentiation, and because very little is known about the molecular biology of smooth muscle growth and differentiation, we attempted to determine whether or not homeobox genes are expressed in vascular smooth muscle and what their role might be in this tissue. Furthermore, because VSMCs undergo migration, changes in their state of differentiation, and increased proliferation (in other words, remodeling) in the human disease atherosclerosis and in the process of arterial restenosis after balloon angioplasty of atherosclerotic lesions [4-6, 8, 10, 12], we thought it likely that the identification and characterization of vascular smooth muscle homeobox genes, whether novel or previously described, would produce new insights into the pathogenesis of these diseases.

**Atherosclerosis, Vascular Smooth Muscle, and Phenotypic Modulation**

As the principle cause of myocardial infarction, strokes, and gangrene of the extremities, atherosclerosis is responsible for nearly half of all mortality in developed
countries, and also results in significant morbidity in terms of loss of limb due to peripheral vascular disease and incapacitation due to nonlethal myocardial infarctions and strokes [58]. The lesions of atherosclerosis, atherosclerotic plaques, were proposed by Ross in 1973 to be the result of an excessive inflammatory fibroproliferative response by VSMCs to chronic injury to the endothelium of the vessel wall [59]. Normally, this response represents a protective mechanism allowing for repair of arterial damage; however, in atherosclerosis, the process becomes chronic, leading to migration of VSMCs from the media into the intima and their subsequent proliferation in response to various growth factors released by endothelial cells, platelets, and macrophages [10-12, 58, 60-62]. Many factors have been implicated in causing or exacerbating this endothelial injury, including hypercholesterolemia and abnormal lipoprotein metabolism [12, 63-66], cigarette smoking [67], turbulent blood flow [68, 69], and hypertension [12, 70]. Eventually, this uncontrolled chronic inflammatory response can lead to impingement on the lumen, compromising blood flow through the vessel. When the lumen narrows to the point that the blood flow through the artery is no longer sufficient for the metabolic needs of the tissue or organ supplied by the affected vessel, or when the endothelial lining of the narrowed lumen becomes thrombogenic due to the underlying inflammatory response, complications result, the nature of which depend upon the specific vessel involved.

Although it is injury to the endothelium that is postulated to be the inciting event in atherosclerosis [11, 12], and although other cell types, most notably macrophages [71, 72], respond to the cytokines and growth factors released by the injured endothelium, it is the smooth muscle cell that is largely responsible for the complications resulting from atherosclerotic lesions, both because of its proliferative response to growth factors and because of its secretion of extracellular matrix [11, 16, 17, 62, 73-77]. Because of the key role of VSMCs in the fibroproliferative component
of the atherosclerotic disease process, factors influencing their growth have been
extensively studied, as have the receptors and intracellular signaling pathways activated
by these factors [12, 74, 78-89]. These factors are released by macrophages and
monocytes, endothelial cells, degranulated platelets, and other cell types, and can either
stimulate or inhibit VSMC proliferation [12, 16, 74, 81, 90, 91]. Comparatively much
less is known about what are presumably the end targets of these pathways: nuclear
transcription factors which activate and/or repress sets of genes necessary for the
transition from the quiescent state to the proliferative state. Identification of such
factors might reveal new targets for intervention in the proliferative process.

VSMCs are mesenchyme-derived cells resembling fibroblasts in embryological
and early postnatal development [80, 92-94]. However, as the animal matures, these
cells gradually develop into cells that are mononucleated and diploid and whose
cytoplasm is full of actin and myosin filaments [95]. The principal morphological
difference between smooth muscle and skeletal or cardiac muscle is that in smooth
muscle these filaments are not arranged in highly ordered sarcomeres, but rather appear
to be almost randomly oriented in the cytoplasm [92, 93]. VSMCs are morphologically
and functionally different at different stages of development as well [3-5, 13, 16, 17,
80, 83, 92]. For example, there are marked differences between fetal, neonatal, and
adult vascular smooth muscle in growth factor response [80], growth factor production
[83], and in the relative expression of different isoforms of actin and myosin [4, 5,
92], for example. The contraction and relaxation of these cells in response to various
agonists is the main determinant of vascular tone and systemic blood pressure and can
also be part of the pathogenesis of many disease processes, ranging from hypertension,
to anaphylactic reactions, to septic shock.

Unlike cardiac and skeletal muscle cells, which are terminally differentiated and
therefore incapable of further cell division, VSMCs retain the ability to re-enter the cell
cycle in response to the appropriate signals. The binding of appropriate growth factors to cell surface receptors initiates a cascade of intracellular signals which not only stimulate the cell to divide, but alter the cell’s state of differentiation [4, 6, 9, 12, 13, 15, 16, 90, 96]. Upon re-entering the cell cycle, VSMCs activate certain genes [97] and down-regulate others, in the process also dedifferentiating and expressing lower levels of smooth muscle-specific isoforms of contractile proteins and higher levels of nonmuscle actins and myosins [6, 8, 9, 15, 77, 90, 96, 98, 99].

Based on cell culture models and in vivo observations, it has been postulated that VSMCs exist within a spectrum of phenotypes ranging from the “contractile” to the “synthetic” state [15, 16, 93, 100]. Cells in the contractile state do not migrate; are relatively insensitive to mitogens; express contractile proteins at a high level, including smooth muscle-specific isoforms of actin and myosin; and are associated with normal vessel wall [15, 16]. These cells also respond to agents that induce vasoconstriction or vasodilation, including catecholamines [101], angiotensin II [76, 79], prostaglandins [102], and others. Synthetic state cells, on the other hand, are able to migrate [16]; express lower levels of contractile proteins, with higher levels of nonmuscle isoforms of myosin and actin [4, 90, 96, 103, 104]; secrete extracellular matrix components [105]; have a smaller percentage of cytoplasm filled with myofibrils [16]; and generally resemble their less differentiated precursors in fetal blood vessels [16, 92, 96]. They are also responsive to growth factors, many of which are not expressed in normal artery but are up-regulated in atherosclerotic plaques, such as platelet-derived growth factor [61, 74], basic fibroblast growth factor [106], insulin-like growth factor 1 [78], interleukin-1 [85, 107], transforming growth factor β [81, 91], and several others [12, 74].

Although the distinction between the contractile and synthetic phenotypes is not absolute and there appears to be overlap between the spectrums of phenotypes...
encompassed by these terms, numerous studies have suggested that the contractile phenotype is associated with normal adult vessel wall, whereas the synthetic phenotype is associated with early development, repair of arterial injuries, VSMC proliferation on prosthetic vascular grafts, atherosclerotic lesions, and other blood vessel diseases where vascular myocyte proliferation is disordered [4-6, 8, 9, 12, 15, 17, 77, 80, 99, 108]. The conversion of a particular cell from one state to the other has been described as "phenotypic modulation" [15, 16], and is observed several days after VSMCs are first plated in primary culture [16, 93]. Several studies suggest that this conversion also takes place in VSMCs that have migrated to the intima and proliferated during the response to arterial injury by endothelial denudation and those found in atherosclerotic lesions [5, 8, 9, 17]. In cell culture models, primary cultures of VSMCs which have been converted to the synthetic phenotype can revert back to the contractile phenotype under certain conditions ("reversible synthetic state cells"). However, after a time that depends both on the period of time since the cell's change to the synthetic state and the number of population doublings undergone by the cells, this change becomes irreversible, and these VSMCs are immediately responsive to serum mitogens upon subculturing ("irreversible synthetic state cells") [16, 100]. It is as yet unclear whether the proliferating VSMCs in atherosclerotic plaques represent reversible or irreversible synthetic state cells, but the phenotypic modulation to the synthetic state in vivo clearly is reversible in acute processes such as the proliferative response of vascular myocytes to acute arterial injury caused by endothelial denudation [16].

This distinction between the two states is useful in considering the coordination of growth and differentiation because of the functional implication: i.e., for the cell to divide, it tends to need to return to a more primitive, less differentiated state, and therefore it is possible, perhaps even likely, that the same transcription factors responsible for activating the proliferative program might also be responsible for
deactivating tissue-specific genes associated with the differentiated state, such as the
smooth muscle-specific forms of actin and myosin. The question then becomes: What
are the transcription factors responsible for regulating the expression of proliferative
and/or tissue-specific genes associated with the differentiated state? It is likely that
such a factor would have several or all of the following characteristics:

1. Its expression is cell type-specific.

2. Its expression is responsive to mitogens and/or the cell cycle.

3. It influences or controls cell growth, differentiation, and/or migration.

4. It is a transcription factor and can activate and/or repress expression of
target genes.

In the search for the identities of factors controlling the growth and
differentiation of VSMCs, it was natural to seek an analogy with skeletal muscle, a
highly related tissue whose developmental molecular biology is much better
characterized than that of smooth muscle. In skeletal muscle, master regulatory genes
capable of activating the myogenic program in nonmuscle cells have been identified and
characterized. These factors are members of the helix-loop-helix (HLH) family of
transcription factors and include *MyoD*, *myf-5*, *myogenin*, and others [20-23]. These
genes, when expressed in non-muscle cells, can promote the development of these cells
into myoblasts and skeletal muscle, simultaneously arresting cell growth [109, 110],
and at least one, *myogenin*, is necessary *in vivo* for normal muscle development [18,
19]. There are yet no known master regulatory genes for smooth muscle, vascular
or other, analogous to *MyoD* or *myogenin* in skeletal muscle, and previous attempts by
this laboratory to isolate HLH transcription factors expressed in VSMCs have not been
successful. Given the ability of VSMCs to reversibly change their state of
differentiation and re-enter the cell cycle, it is possible that the VSMC counterparts of
skeletal muscle master regulatory genes will not be as easy to identify, because it is
probable that they would not have as dramatic an effect on cellular phenotype as MyoD and other HLH factors when overexpressed in nonmuscle cells. The plasticity of the smooth muscle phenotype and its resemblance during proliferation to that of a fibroblast (at least under light microscopy) [93] stands in marked contrast to that of the skeletal muscle cell, which cannot divide again and has a very distinctive phenotype. However, it is likely that there exist smooth muscle transcription factors which regulate or influence phenotypic modulation of VSMCs, activating and repressing batteries of subordinate genes appropriate to the contractile or synthetic phenotypes in response to signals carried to the nucleus by intracellular signaling pathways from growth factor receptors on the cell surface. Based on their critical roles in such diverse processes as cell growth control, pattern formation, organogenesis, and cell migration [24-57], we postulated that homeobox genes were good candidates either for being VSMC master regulatory genes or for regulating phenotypic modulation in vascular cells. In the following section, the properties of homeobox genes which make them good candidates for major regulators of VSMC proliferation and differentiation and potential regulators of phenotypic modulation are discussed in more detail.

**Homeobox Genes in the Cardiovascular System**

Homeobox genes were first characterized in the study of homeotic mutations in *Drosophila melanogaster*. In a homeotic mutation, one body part or segment is replaced with another, normally formed segment. The prototypical example is the *Drosophila* gene *antennapedia*, mutations of which can cause the replacement of the fly’s antennae with normally formed legs. Isolation and characterization of many of the genes responsible for these mutations revealed that they encode transcription factors with a common 61 amino acid DNA-binding motif, the homeodomain, containing a helix-turn-helix motif similar to that found in prokaryotic regulatory proteins such as
Cro, CAP, and the λ repressor in *Escherichia coli* [34, 55]. Because several homeodomain-containing proteins in *Drosophila* are critical in morphogenensis and cellular differentiation [27, 38, 45, 49, 55, 111, 112], much effort was expended to discover and characterize their vertebrate counterparts. As a result, many homeobox genes closely related to the first known *Drosophila* homeoboxes have been found to be expressed in many invertebrates, invertebrates, and even plants, suggesting that they are likely to be ubiquitous [25, 26, 35, 45, 49-51, 53, 55, 113-128]. In vertebrates, these genes often share similar functions to their *Drosophila* homologues, and can in some cases substitute for them functionally in development when expressed in *Drosophila* embryos [129]. In both *Drosophila* and vertebrates, many, but not all, homeobox genes are arranged in clusters [25, 39, 41, 45, 49, 55]. In *Drosophila* there is one major homeotic complex (HOM-C), and in mice and humans there are four unlinked complexes, HOX-1 through HOX-4 (also referred to as HOX A through HOX D)\(^1\). The *Drosophila* homeotic genes map in the same order in the HOM-C as their functional domains lie on the embryo's anterior-posterior axis. This same organization is shared by the four vertebrate HOX clusters, where gene order in the cluster corresponds to the relative anterior-posterior boundaries of expression and the temporal order of activation during embryogenesis [39, 41, 49].

Evidence for the importance of homeobox genes in specifying body plan and cell fate in vertebrates during embryogenesis includes the demonstration of segmental defects or homeotic mutations in transgenic mice homozygous for null mutations of

---

\(^1\) Recently, a change in homeobox gene nomenclature has been proposed, in order to make the naming of these genes more systematic [130]. Because this change is recent and the older names for these genes are still widely used by many investigators, we use the older nomenclature here. However, the new name appears in parentheses, if such a designation exists, when the gene is initially listed in this thesis, and a list of the old names with their corresponding names according to the new nomenclature appears in Appendix I.
these genes which correspond to their spatial domain of expression [29, 30, 46, 48]. Several vertebrate homeobox proteins are transcription factors which regulate expression of lineage-specific genes and can control cell differentiation [26-30, 40, 46, 48, 54, 120]. Examples from the POU subclass of homeobox proteins include Oct-2, whose overexpression is sufficient for specific activation of gene expression by B-lymphocyte-specific promoters in non-B-cells [51], and GHFI (also known as Pit-1), which activates pituitary-specific genes such as those for prolactin and growth hormone and is necessary for the growth of the cell types producing these hormones [28, 40, 47]. Since homeobox genes control body plan formation during development, it is not surprising that they also influence cell migration in addition to cell differentiation. Recent evidence that homeobox factors directly control cell migration comes from studies where the injection of the goosecoid homeobox mRNA into Xenopus embryonic cells led the induction of region-specific cell migration during gastrulation [52]. Similarly, the mab-5 homeobox gene has been shown to control neuronal precursor cell migration during Caenorhabditis elegans development [131].

Homeodomain proteins can directly control cell proliferation as well. Recently, it has been convincingly shown that some homeobox genes are oncogenes [24, 33, 42, 56]. This was first suspected on the basis of observations that some human leukemias were associated with translocations causing unregulated expression of homeobox genes or fusions of homeobox genes with other genes. Examples include Pbx1 and Hox-2.4 (Hoxb-8) [31, 42, 44, 132], and overexpression of either of these genes was found to be able to transform cells, enabling them to form tumors in nude mice [24, 42, 132]. Other homeobox genes which have recently been shown to be oncogenes include the human gene HB24, which has been associated with acute myelogenous leukemia [33], and the mouse homeobox gene Hox-7.1 (msx-1) [56]. Of particular note, Hox-7.1 is expressed in the proliferative cells of the endocardial cushion that give rise to the
Septum and the valves of the heart [133]. Since regulation of cell growth and of cell differentiation are generally dependent upon each other, it is not surprising that these oncogenic homeobox genes also appear to regulate the state of cell differentiation, as in the case of Hox-7.1 or HB24, with expression of these genes tending to favor a more undifferentiated state [33, 56]. Other homeobox genes, such as GHF1 and Oct-2 tend to promote a more differentiated phenotype and activate the expression of tissue-specific genes [54]. Thus, homeobox genes truly have pleiotropic roles in many cell types and can promote both cell differentiation and cell de-differentiation, as well as control cell growth and migration [24-31, 33, 34, 37-52, 54-57]. Collectively, these features make homeobox proteins promising candidates as regulators of cellular differentiation and remodeling in the cardiovascular system during normal development and in pathological states.
Figure 1. Expression of HOX-1 cluster members in the developing embryo. The spatial expression pattern of Hox-1.5, Hox-1.6, and Hox-1.11 along the embryonic body axis is shown in a mid-development embryo relative to the order of these genes on the chromosome and relative to the developing heart and vascular system. The anterior most boundary of expression for Hox-1.11 is within branchial arch 2; for Hox-1.6, between branchial arches 2 and 3, and for Hox-1.5, between branchial arch 3 and 4. Generally, these genes display a sharply demarcated anterior boundary of expression, but expression diminishes in more posterior positions. Mice transgenic for null mutations in Hox-1.5 show profound cardiovascular anomalies; where as Hox-1.6−/Hox-1.6− mice do not. The phenotype of Hox-1.11−/Hox-1.11− mice is unknown.
Tinman: A Regulator of Heart Development in Drosophila

The importance of homeobox transcription factors in the cardiovascular system is demonstrated by studies of a Drosophila gene dubbed tinman because mutations within this locus give rise to flies that have no heart [27]. In Drosophila the circulatory system is “open” in that the internal organs are bathed in the blood or hemolymph that is propelled by a four-chambered tube (heart) consisting of a single striated muscle layer. Tinman, previously referred to as msh-2, is expressed in the mesoderm destined to become the heart, and, later in development, expression is completely confined to the heart [27, 134, 135]. In contrast, other homeobox genes, such as apterous, S59 and msh-1, are expressed in the striated muscles of the body wall that derive from somites (skeletal muscle) [133, 136, 137]. Embryos with tinman null mutations also lack the visceral muscles that comprise the gut (smooth muscle), but the somite-derived musculature is relatively normal [27]. These findings demonstrate similarities in the molecular pathways of cardiac and smooth muscle development that differ from the mechanisms controlling skeletal muscle development from somites.

HOX-1 Cluster Members in the Vertebrate Cardiovascular System

Several members of the HOX-1 cluster are expressed in the cardiovascular system during embryogenesis and their expression persists after birth. Homeobox sequences isolated from an adult rat vascular smooth muscle cDNA library² include Hox-1.11 (Hoxa-2), Hox-1.4 (Hoxa-4), and Hox-1.3 (Hoxa-5) [35, 53]. In early development these genes are expressed along the anterior-posterior axis with anterior boundaries that correspond to the gene order in the cluster (Figure 1) [49]. Little is known about the potential roles of Hox-1.3 and Hox-1.4 in regulating gene expression.

²The details of the isolation of these cDNAs are discussed further in Chapters 2 and 3.
in the cardiovascular system, but *Hox-1.11* has a pattern of expression in the cardiovascular system of the developing embryo which suggests its importance in cardiovascular development [53]. By *in situ* hybridization, in early embryogenesis *Hox-1.11* is widely expressed in many tissues of both mesodermal and ectodermal origin, but becomes progressively more confined to the vascular system as development proceeds. Throughout embryogenesis and in neonates, it is expressed in the ventricular cardiomyocytes; however, shortly after birth, its expression in cardiomyocytes ceases, and it is undetectable in adult heart [53]. When the lungs begin to function at birth, there is a marked increase in afterload on the left ventricle and pulmonary blood flow as the circulatory system switches from its fetal configuration to its normal configuration, and this leads to numerous alterations in cardiomyocytes at the molecular level [7, 138]. The transient expression of *Hox-1.11* in the neonatal heart suggests that it may participate in remodeling of the cardiovascular system that occurs at birth.

Functional evidence for the importance of a homeobox gene in the development of the cardiovascular system exists for *Hox-1.5* (*Hoxa-3*). Transgenic mice homozygous for null mutations of this gene die shortly after birth, presumably of cardiac dysfunction due to multiple defects in the cardiovascular system, which include dilation and thickening of the wall of the right atrium and left ventricle and an abnormally small and thin-walled right ventricle [29]. Other prominent defects in the hearts of these mice included hypertrophy of both atria, persistent patent ductus arteriosus, and stenosis of the aortic valve. In the vascular system, lack of a right carotid artery was observed in some mutant mice, and in all mice the aorta had a thin wall and was poorly developed. The homozygotes were also athymic, lacked parathyroids, had reduced thyroid and submaxillary tissue, and demonstrated a number
of craniofacial defects, with the overall constellation of defects resembling the pathology of the human congenital disorder DiGeorge’s syndrome. An interesting finding made with the Hox-1.5−/Hox-1.5− mutation is that the defects found in the transgenic mice did not simply correlate with the pattern of Hox-1.5 gene expression in normal mouse embryos. Tissues that express Hox-1.5 during embryogenesis yet show no defects in the homozygous mice include components of the nervous system, lung, stomach, spleen and kidneys. Presumably, the lack of a mutant phenotype in these organs results from functional redundancies between Hox-1.5 and other regulatory molecules. However, the phenotypes of the Hox-1.5−/Hox-1.5− mice demonstrate that this factor is a regulator of heart and blood vessel development, and that other developmental pathways either do not exist or are insufficient to compensate for the loss of Hox-1.5 in the cardiovascular system. Moreover, the consensus binding site DNA sequence for Hox-1.5 has recently been determined [139], and this information will permit the identification of its downstream target genes.

In comparison, mice transgenic for a null mutation in Hox-1.6 (Hoxa-1), which is closely linked to Hox 1.5, demonstrate no significant cardiovascular anomalies, although they do demonstrate abnormalities in inner ear and hindbrain development [30, 48]. Given that Hox-1.11 maps to a locus located between Hox-1.6 and Hox-1.5 in the Hox-1 cluster (see Figure 1), and given how genes in the Hox clusters control cell fate along the anterior-posterior axis corresponding to their order on the chromosome, the phenotype of a mouse transgenic for a null mutation in Hox-1.11 will be of great interest.

**Gax: A Growth Arrest Specific Homeobox Gene in Vascular Smooth Muscle**

Recently, a tissue-specific diverged homeobox cDNA was isolated from adult rat vascular smooth muscle. Gax [35] is unique in that its expression is regulated like
that of a growth arrest-specific (gas) or growth arrest and DNA damage-inducible (gadd) genes [140-149]; that is, its expression is slowly induced by signals leading to growth arrest and is rapidly down-regulated when quiescent VSMCs are stimulated to re-enter the cell cycle with mitogens. Details regarding the cloning of Gas and the regulation of its expression will be discussed in detail in Chapters 2 and 3.

**MHOX/PHOX**

Recently, a diverged homeobox gene showing a high degree of specificity to muscle, including cardiac and smooth muscle, has been isolated from both human and mouse cDNA libraries by different techniques [37, 117]. The gene, whose mouse homologue is known as *MHOX* and whose human homologue is known as *PHOX*, was isolated by two groups using two entirely different approaches. *MHOX* was isolated from a C2 myoblast cDNA library by its ability to bind an A/T-rich sequence in the muscle creatine kinase (MCK) enhancer that is essential for muscle-specific gene transcription and trans-activation by myogenic basic region-helix-loop-helix (bHLH) proteins [117]. *PHOX*, on the other hand, was isolated from a human glioblastoma cDNA library by its ability to complement mutants deficient in a yeast gene which normally interacts with the yeast homologue of the serum response factor (SRF) [37]. Its homeodomain was then shown to be able to enhance the DNA-binding activity of the SRF for the serum response element (SRE) by a mechanism independent of DNA binding by the homeodomain.

The SRE has the core sequence of CC(A/T)GG, or CArG motif, which is found in the promoters of immediate early genes, such as *c-fos*, and in the promoters of many muscle-specific genes, including the actins, MCK, and dystrophin. These elements are multifunctional in that they confer the rapid and transient activation of expression of immediate early genes in response to mitogen stimulation, and they also
have critical regulatory roles in some muscle-specific genes. Notably, the proximal CArG motif of the skeletal actin promoter is sufficient for muscle specific expression upstream from the TATA box of a non-muscle gene [150]. This element mediates the upregulation of skeletal actin expression in cardiomyocytes in response to basic fibroblast growth factor, and thus, may contribute to the aberrant re-expression of this gene during cardiac hypertrophy [151].

The reported interaction of Mhox/Phox with the SRF at CArG elements is particularly significant because it implies another mechanism by which homeobox genes could potentially regulate gene expression [37]. Instead of directly binding to DNA regulatory elements, certain homeobox genes could interact with other transcription factors and thereby modulate their activity. Furthermore, the reported interaction of Mhox/Phox with an essential A/T-rich element in the MCK enhancer [117] establishes precedence for the involvement of homeodomain proteins in myocyte-specific gene transcription. In the case of the MCK gene, the A/T-rich homeodomain binding site is required in conjunction with the binding sites for myogenic bHLH proteins for high levels of expression, suggesting a possible functional interaction between these two classes of developmental regulatory molecules. A similar regulatory mechanism exists for the insulin gene in pancreatic β-cells, where the homeodomain protein Imx-1 and the HLH protein ShPan-1 bind to the enhancer and synergistically activate its expression [152].

**Conclusions**

Until recently, little was known about tissue-specific transcription factors that regulate gene expression in the cardiovascular system. Because of the widespread role of homeobox genes in regulating the growth, differentiation, and migration of diverse cell types in both vertebrates and invertebrates [24-57], it was logical to hypothesize
that they might play such a role in the cardiovascular system as well, especially considering the tissue remodeling and molecular alterations associated with pathology and normal development of the heart and blood vessels. To date, homeobox genes directly or indirectly implicated in the regulatory control in the vertebrate cardiovascular system include Gax, MHox/Phox and members of the Hox-1 cluster including Hox-1.5 and Hox-1.11 [29, 35, 37, 53, 117]. The regulatory roles of other homeobox genes known to be expressed in the cardiovascular system, such as Hox-1.3 and Hox-1.4, remain to be elucidated. It may be that they are redundant, as Hox-1.5 appears to be in the parts of the central nervous system in which it is expressed, or it may be that they, too, have important developmental roles. Also, it is unlikely that the homeobox genes isolated from the cardiovascular system thus far are the only ones expressed there, and it is probable that many more will be found. Indeed, recently a novel tissue-specific homeobox gene, Csx for Cardiac-Specific homeoboX, was identified that is exclusively expressed in the developing and adult heart (S. Izumo, personal communication).

Most studies to date on homeobox genes in vertebrates have focused on elucidating their expression patterns during embryogenesis, and function was inferred by analogy to work in experimental organisms more amenable to genetic manipulations. Most notable is the work on the tinman homeobox gene in Drosophila which specifies heart and visceral muscle development [27]. However, important roles for homeobox genes in the vertebrate cardiovascular system have been revealed by recent advances in transgenic mouse technology, as with Hox-1.5, and by advances in the understanding of transcriptional control through the use of yeast selection and expression library screening techniques, as with MHox/Phox.

The most important challenge remaining in understanding the roles of homeobox transcription factors is to determine which of these genes influence the
growth and differentiation of these tissues, since the study of these genes will provide clues about normal and abnormal development. For example, in the vascular system such a gene might prove to be a potential target for gene therapy of diseases such as coronary artery restenosis or atherosclerosis, a prominent feature of whose pathophysiology includes the disordered growth of VSMCs. This will prove especially valid if this gene shows more tissue specificity than other such targets (for example, proto-oncogenes). To this end, we attempted to identify which, if any, homeobox genes are expressed in VSMCs by screening a rat aorta cDNA library with degenerate oligonucleotides designed to hybridize with the most conserved region of the antennapedia homeodomain. As a result of this study, several previously characterized homeobox genes of the HOX-1 cluster were found to be expressed in vascular smooth muscle [35, 53], as well as at least one previously unreported gene, Gax, whose expression was found to be regulated according to the cell cycle [35] and whose characteristics are discussed in detail in Chapters 2 and three. Also, we determined that another homeobox gene, Mhox/Phox, which has been shown to interact with DNA regulatory elements essential for muscle-specific gene expression in skeletal muscle [117] and to enhance the binding of the serum response factor for the serum response element [37], is also regulated according to the cell cycle in VSMCs, but in the opposite direction. Details of this work will be discussed in Chapter 5. Finally, we investigated whether any of these genes has a direct effect on vascular cell growth by microinjecting recombinant protein into smooth muscle cells to see if growth was suppressed, and found that Gax, as we predicted by its expression pattern, was able to inhibit the entry of quiescent VSMCs into S-phase (see Chapter 6), thus demonstrating that homeobox genes do play an important role in regulating the growth of vascular smooth muscle.
References


muscle cells is phenotype and growth state dependent. *Growth Factors* 3: 191-203.


CHAPTER 2:

CLONING AND SEQUENCING OF HOMEBOX cDNAs FROM ADULT RAT AORTA USING A DEGENERATE Oligonucleotide PROBE CONTAINING INOSINE RESIDUES

Introduction

Screening cDNA libraries by hybridization with radioactively labeled oligonucleotides designed to recognize highly conserved sequences has been a mainstay of molecular biology for several years, and is a technique which has been used to isolate many cDNAs on the basis of their homology to known proteins or genes. This technique has been widely used in the isolation of homeobox genes because sequences within the 180 base pair homeodomain are highly conserved [1-3]. The identification of the homeobox genes expressed in a particular cell type is desirable because these molecules have key roles in the determination of cell identity. Short, degenerate oligonucleotides have been used by several investigators to isolate novel homeobox cDNAs from various libraries [4-12] and in a screen of a low complexity genome it was reportedly possible to derive initial DNA sequence utilizing the same highly degenerate oligonucleotide probe that was used to screen the cDNA library as a sequencing primer [5], although neither the quality nor the quantity of this sequence information was reported. In general, difficulties arise in designing probes for homeodomain sequences, because the most highly conserved region encodes two arginine residues [3], each of which can be represented by six different codons, necessitating highly degenerate probes. Typically such screens utilize short (15- to
25-mers), highly degenerate (512- to 2048-fold) oligonucleotides [5-8], which makes it difficult or impossible for subsequent sequence analysis using the screening probe. The advantage of using the screening probe for sequence determination is that the identity of the clone can be determined rapidly from the nucleotide sequence adjacent to the site of probe hybridization rather than sequencing hundreds, or even thousands, of bases into the cDNA inserts from known vector sequences. Here we describe a procedure that utilizes the same deoxyinosine-containing, low degeneracy oligonucleotide to screen a complex cDNA library and to rapidly screen for clones containing homeodomains by sequencing the clones with the same oligonucleotide as a primer. With this procedure it was possible to isolate several homeobox cDNAs from a screen of a complex vertebrate cDNA library and to derive high quality sequence from all homeobox-containing clones isolated. The principal advantage of this technique over standard screens of cDNA libraries with highly degenerate oligonucleotides is that it permits the rapid screening of a complex cDNA library and the ability to determine quickly which clones are worth further study.

Materials and Methods

Oligonucleotide Design and Initial DNA Database Homology Searches

A 64-fold degenerate 29-mer oligonucleotide containing 3 inosine residues, designated H3I (see Figure 2A), was synthesized by the phosphoramidite method on an Applied Biosystems 391 EP oligonucleotide synthesizer. To test the specificity of this oligonucleotide, its sequence was used for searches versus the Genbank database to find what sequences have a sufficiently high degree of homology to the probe such that they would be likely to hybridize to it. Searches were carried out using a Macintosh Quadra 800 computer running MacVector DNA sequence analysis software (version 4.0.1) versus the Genbank database on CD-ROM (version 71.0). MacVector
A. Design of H3I Probe

\[
\begin{array}{cccccccccc}
\text{K} & \text{I} & \text{W} & \text{F} & \text{Q} & \text{N} & \text{R} & \text{R} & \text{M} & \text{K} \\
\text{AA} & \text{AT} & \text{TGG} & \text{TT} & \text{CA} & \text{AA} & \text{AG} & \text{AG} & \text{ATG} & \text{AA} \\
\end{array}
\]

5'- AA AT TGG TT CA AA AG AG ATG AA 3'

B. Alignments of cDNAs isolated

<table>
<thead>
<tr>
<th>H3I</th>
<th>5'- AA</th>
<th>AT</th>
<th>TGG</th>
<th>TT</th>
<th>CA</th>
<th>AA</th>
<th>AG</th>
<th>AG</th>
<th>ATG</th>
<th>AA-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hox-1.3</td>
<td>AAA</td>
<td>ATC</td>
<td>TGG</td>
<td>TTC</td>
<td>CAA</td>
<td>AAC</td>
<td>AGG</td>
<td>AGG</td>
<td>ATG</td>
<td>AA</td>
</tr>
<tr>
<td>Hox-1.4</td>
<td>AAG</td>
<td>ATC</td>
<td>TGG</td>
<td>TTC</td>
<td>CAG</td>
<td>AAC</td>
<td>CAG</td>
<td>AGA</td>
<td>ATG</td>
<td>AA</td>
</tr>
<tr>
<td>Hox-1.11</td>
<td>AAA</td>
<td>GTG</td>
<td>TGG</td>
<td>TTT</td>
<td>CAG</td>
<td>AAC</td>
<td>CAG</td>
<td>AGG</td>
<td>ATG</td>
<td>AA</td>
</tr>
<tr>
<td>Hox-2.3</td>
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<td>TGG</td>
<td>TTT</td>
<td>CAG</td>
<td>AAC</td>
<td>CGG</td>
<td>CGC</td>
<td>ATG</td>
<td>AA</td>
</tr>
<tr>
<td>Gax</td>
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<td>TGG</td>
<td>TTC</td>
<td>CAG</td>
<td>AAC</td>
<td>AGG</td>
<td>AGA</td>
<td>ATG</td>
<td>AA</td>
</tr>
</tbody>
</table>

Figure 2: H3I probe sequence aligned with sequences isolated from the rat aorta cDNA library. A. Peptide sequence of the highly conserved third helix of the Antennapedia homeodomain, the translation of this sequence, and the sequence of the H3I probe. B. Homeodomain sequences isolated from the rat cDNA library aligned with the H3I probe. Mismatched bases are underlined. None of the clones isolated had more than one mismatch.

uses the Lipman/Pearson algorithm to find and score matches [13], and a hash value of 3 was used for all searches. In addition, further searches were done by sending the sequence by electronic mail via the Internet to blast@ncbi.nlm.nih.gov, which utilizes the BLAST algorithm [14, 15] to search the latest versions of the Genbank and EMBL databases.

cDNA Library Screening and Sequencing of Clones Isolated

An adult rat aorta cDNA library [16] in \( \lambda \) ZAP was screened with the H3I oligonucleotide described above. 500,000 recombinant phage colonies in \( E. \ coli \) were adsorbed in duplicate to nitrocellulose membranes and hybridized at 42° C with this oligonucleotide end-labeled with \( \gamma^{32}\text{P-} \)ATP in 0.5 M sodium phosphate (pH 7.0),
7% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 1% bovine serum albumin [17]. The filters were washed to a final stringency of 0.5X SSC (1X SSC = 150 mM NaCl, 26 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 with Sequenase® v.2.0 (United States Biochemicals) according to the instructions of the manufacturer. Initial sequencing attempts were performed using the same degenerate oligonucleotide used to screen the cDNA library. 5 μg of template DNA were used. For nondegenerate oligonucleotide primers, 2 pmol per reaction were used; for HB3I, 128 pmol per reaction were used.

Results and Discussion

Design and predicted hybridization characteristics of the H3I probe

It is possible to overcome the limitations of probe degeneracy through the use of oligonucleotides containing deoxyinosine residues. Deoxyinosine has been found to be useful in reducing the specificity of DNA probes, especially at positions containing three- or four-fold nucleotide ambiguities, because of its lack of specificity in Watson-Crick base-pairing relative the four normally occurring bases, which makes it, in effect, a “neutral” base, able to base-pair with deoxyadenosine, deoxycytidine, deoxyguanosine, or deoxythymidine [18, 19]. This relative neutrality with regard to base-pairing also increases the effective probe concentration, because a probe containing a deoxyinosine residue at a highly ambiguous position will be able to hybridize with all possible nucleotides in that position. In contrast, in the case of mixtures of oligonucleotides, each containing a different base at the position of ambiguity, only the fraction of probe containing the correct base at the ambiguous
position will be able to hybridize effectively. Thus we reasoned that an oligonucleotide probe with deoxyinosine residues would be ideal in screens for homeobox cDNAs because it would permit the same oligonucleotide to serve both as a probe to screen the cDNA library and as a primer for sequencing the clones isolated.

A 64-fold degenerate 29-mer oligonucleotide (designated H3I) containing deoxyinosine residues was designed to recognize the most highly conserved region of the homeodomain, helix 3. (See Figure 2A for probe design and sequence.) Deoxyinosines were incorporated at codon positions of maximum ambiguity, where the genetic code could be satisfied by three or more different bases. In order to test whether the sequence of H3I would be likely to be able to discriminate between homeodomain cDNAs and other cDNAs and to obtain a general idea of what nonhomeobox cDNAs might potentially be isolated using H3I, we performed homology searches versus the Genbank and EMBL databases. First, we used MacVector DNA sequence analysis software to search Genbank on CD-ROM, as described in Materials and Methods, and the 200 highest scoring matches were retained. Of these matches, 200/200 (100%) represented homeobox genes, indicating that, on homology searches at least, H3I demonstrates a high degree of specificity for homeobox sequences. Sequences retained included homeoboxes from several different classes: Antennapedia, Deformed, labial, Abdominal B, eve, prd, Hox-1.5, Hox-2.4 [3]. Homeoboxes from several different species matched as well, including mouse, human, bee, cat, chicken, D. melanogaster, rat, newt, salmon, C. elegans, and X. laevis, among others. No members of the POU class of homeobox genes were in the top 200, indicating that the probe was less likely to recognize this class. Of these, 175/200 (87.5%) had zero base mismatches, 15/200 (7.5%) had one mismatch, and 10/200 (5%) had two mismatches. (Note that deoxyinosines were considered to match any base for purposes of this analysis.) Because of a limitation in MacVector,
it was not possible to list more than 200 of the top-scoring matches and alignments in the homology search. Therefore, in order to determine how many total homeobox sequences might be recognized by this probe and evaluate the possibility that non-homeobox sequences might be recognized, we performed homology searches for H3I versus the latest versions of the Genbank and EMBL databases using the BLAST algorithm [14, 15]. Of the highest scoring sequences, 631/631 (100%) were homeobox sequences. The highest-scoring non-homeobox gene in the database was the rat Cyp4a locus, encoding cytochrome P450 (IVA3), but optimal alignment of this sequence with the H3I 29-mer produced 7 mismatches. Thus it would be unlikely that this cDNA would be recognized by our inosine probe, even at low stringency.

Finally, an analysis of 72 classified homeodomain peptides [3] revealed that greater than 90% would be recognized by the H3I probe allowing none or one mismatch, and only the homeodomains classified as Paired or POU would not be predicted to hybridize to the H3I probe. Thus, on the basis of homology searches, we expected that H3I would be highly specific to eight of ten classes of homeobox sequences [3], even when used for screening complex cDNA libraries. We note, however, that others have utilized two different inosine-containing probes, one to the POU homeodomain and one to the POU-specific domain, to screen a cDNA library for members of this homeobox class [10].
Figure 3: Examples of sequence obtained using the degenerate oligonucleotide H3I as a primer. Sequencing reactions were separated on a 6% polyacrylamide gel with 50% urea, and the xylene cyanole marker band was run to the bottom of the gel. From this gel, it was possible to read with reasonable accuracy 277 bases of Gax sequence, 280 bases of Hox-1.3 sequence, and 309 bases of Hox-1.4 sequence. For Hox 2.3, a short partial cDNA was isolated, and 48 bases of its sequence could be clearly read on this gel prior to Bluescript vector sequence.
Isolation of homeobox cDNA clones from a rat library

In order to isolate cDNAs encoding homeobox proteins expressed in vascular smooth muscle, a λ ZAP cDNA library [16] was screened with H3I at low stringency as described in Materials and Methods. From 500,000 plaques, thirteen positive clones were isolated and the plasmids were excised by standard procedures [20]. In order to determine rapidly which clones contained homeodomains, attempts were made to immediately sequence all clones isolated with the H3I oligonucleotide. High quality DNA sequence information was obtained by standard dideoxy sequencing for 12/13 clones (for an example of the quality of sequence obtained, see Figure 3).

From these sequence data we could deduce that the 12 clones represented transcripts from five different rat genes, including: Hox-1.3, Hox-1.4, Hox-1.11, and Hox-2.3. Upon further sequence analysis it was determined that the fifth class of clones represented a novel homeobox gene that was designated Gax [21]. Collectively, these cDNAs represent members of the Antennapedia class (Hox-2.3), Dfd class (Hox-1.3 and -1.4), and unclassified or diverged homeoboxes (Hox-1.11 and Gax) [20, 22].

The sequences of all of these clones aligned with the probe sequence of H3I are shown in Figure 2B, with mismatches indicated. In the case of the single remaining clone, double-priming occurred with the H3I oligonucleotide, and no useful sequence information could be obtained (not shown). Upon further sequence analysis by walking in from known vector sequences, it was determined that this clone did not contain a homeodomain. Thus, the ability to obtain sequence information from H3I served as a rapid means of screening which clones were novel or of particular interest otherwise. The utility of this method is particularly evident with the Gax cDNA clone, which has a very long 3' untranslated region, and whose homeodomain is located near the C-terminus of the peptide, very near to the center of the cDNA [20]. To determine
the homeodomain sequence of this clone would have necessitated sequencing in from
the vector’s T3 and T7 promoters, a distance of several hundred bases, which would
have required at least three primers to cover. We also note that there is no reason that
a probe designed to sequence toward the 5' end of the cDNA should not be equally
effective, both for screening the library and for use as a sequencing primer. Because
many homeobox genes share identical homeodomains [1, 3, 23] and because we were
interested in rapidly identifying novel cDNAs, we chose to design our primer to
sequence towards the 3' end of the cDNA, in order to sequence the 3' end of the
homeodomain as well as some non-homeodomain sequence on the C-terminal side
with a single primer.

In summary, this protocol offers a significant improvement over previous
homeobox screening protocols with oligonucleotides [4-12] because the same
inosine-containing oligonucleotide is used to screen the library and to sequence the
positive clones. The inosine substitutions are advantageous because they decrease the
degeneracy of the probe without compromising hybridization specificity such that the
probe can be used as an effective primer for dideoxy sequencing. By determining the
sequence of nucleotides immediately adjacent to the site of probe hybridization, the
investigator is rapidly provided with information that can be used to identify which
clones contain known homeobox gene sequences, and this allows the investigator to
decide which clones are most important for further investigation.

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CHAPTER 3:

MOLECULAR CLONING OF A DIVERGED HOMEBOX GENE THAT IS RAPIDLY DOWN-REGULATED DURING THE G₀/G₁ TRANSITION IN VASCULAR SMOOTH MUSCLE CELLS

Introduction

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 re-entering the cell cycle [1-14]. Upon entering the cell cycle, these cells activate certain genes, such as ornithine decarboxylase [15], and down-regulate others, such as smooth muscle-specific α-actin [2, 5, 6, 16]. 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 [10, 11, 13, 17]. Determination of what nuclear factors might potentially 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 G₀, growth and cell division is at a halt, but growth factor binding to receptors initiates a complex series of events causing cells to enter G₁, culminating
with DNA replication during 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 [18, 19]. Potential negative regulators of this transition include the growth arrest-specific (gas) and growth arrest and DNA damage-inducible (gadd) genes [20-31]. These genes are expressed at high levels in quiescent NIH3T3 fibroblasts or CHO cells, but fall dramatically upon mitogen stimulation, and their expression is slowly induced, usually over a period of 12-24 hours by signals causing growth arrest.

Overexpression of one gas gene encoding an integral membrane protein (gas1) inhibits the entry of quiescent fibroblasts into S-phase, suggesting that it has a role in the maintenance of the quiescent state [24], although the mechanism by which this regulation occurs has not yet been determined. One of the gadd genes, gadd 153, is a CCAAT/enhancer-binding protein (C/EBP)-related gene and likely represents the hamster homologue of CHOP-10, a gene whose product is capable of dimerizing with other members of the C/EBP family through its leucine zipper motif and inactivating them [32]. Another gadd gene, gadd45, has been implicated in a mammalian cell cycle checkpoint pathway involving p53, and this pathway is defective in ataxiatelangiectasia [33]. Undoubtedly other genes inhibit the entry of cells into the cell cycle.

Homeobox genes represent a class of transcription factors which, while long known to be important in cell differentiation and growth during embryogenesis, have only yielded 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 A/T-rich consensus sequences with high affinity [34-38]. 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 [39].

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 [35-64]. Examples of this regulation are found in the pituitary (GHF-1/pit-1) [44] and the immune system (oct-2) [61], 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 [40, 47, 48, 56, 63]. In spite of this involvement in differentiation and growth in a wide variety of tissues [35-64], little is known about the regulation of homeobox genes by peptide growth factors, especially in mammalian cells. Based on 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 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 re-enter the cell cycle upon mitogen stimulation. Unlike skeletal muscle, where nuclear factors controlling myogenesis have been much more extensively studied [65-69], 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 potentially regulate its proliferation [70]. 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 re-enter the cell cycle, Gax expression is rapidly down-regulated with a time course similar to that of previously described gas genes [22-24], and the extent of this down-regulation is correlated to the mitogen's ability to stimulate \(^{3}\text{H}\)-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 re-enter the cell cycle.

Materials and Methods

Hybridizations and cDNA cloning

An adult rat aorta cDNA library [71] in \(\lambda\) 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 (also, see Figure 2):

\[
5'\text{-AA(A/G)ATITGGTT(T/C)CA(A/G)AA(C/T)(A/C)GI(A/C)GIATGAA}-3' \\
(I = \text{inosine})
\]

500,000 recombinant phage colonies in \(E. \text{coli}\) were adsorbed in duplicate to nitrocellulose membranes and hybridized at \(42^\circ\text{C}\) with this oligonucleotide end-labeled with \(\gamma^{32}\text{P}\)-ATP in 0.5 M sodium phosphate (pH 7.0), 7% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 1% bovine serum albumin [72]. The filters were washed to a final stringency of 0.5X SSC (1X SSC = 150 mM NaCl, 26 mM sodium citrate, pH 7.0), 0.1% SDS at \(42^\circ\text{C}\), and exposed to X-ray film. Positive signals were isolated and rescreened until the clones were plaque purified. The plasmids containing the clones in \(\lambda\) ZAP vector were then excised by the protocol recommended by the manufacturer (Stratagene), and sequenced on both strands with Sequenase\(^\text{®}\)
v.2.0 (United States Biochemicals). Homology searches were performed versus the Genbank™ and EMBL databases (v.73) using the BLAST algorithm [73].

Interspecific Backcross Mapping

Interspecific backcross progeny were generated\(^1\) by mating (C57BL/6J X M. spretus)F\(_1\) females and C57BL/6J males as described [75]. A total of 205 N\(_2\) mice were used to map the Gax locus. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described [76]. All blots were prepared with Zetabind nylon membrane (AMF-Cuno). The probe, a 1155 bp rat cDNA clone, was labeled with \(\alpha\)-\(^{32}\)P-dCTP using a random prime labeling kit (Amersham); washing was done to a final stringency of 0.2 X SSCP, 0.1% SDS, 65\(^\circ\) C. A major fragment of 4.2 kb was detected in HincII digested C57BL/6J DNA and major fragments of 3.6 and 2.7 kb were detected in HincII digested M. spretus DNA. The 3.6 and 2.7 kb M. spretus-specific HincII fragments cosegregated and were followed in backcross mice.

A description of the probes and RFLP's for the loci linked to Gax including neuroblastoma myc-related oncogene-1 (Nmyc-1), laminin B1 subunit (Lamb-1), DNA segment, Chr. 12, Nyu 1 (D12Nyu1), and β-spectrin (Spnb-1) has been reported previously [77, 78]. Recombination distances were calculated as described [79] using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

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\(^1\)The chromosomal localization of Gax in the mouse genome was determined by Copeland and Jenkins as part of this project [74].
Reagents and cell culture

Recombinant platelet-derived growth factor-AA, -AB, and -BB were obtained from Boehringer-Mannheim, and PDGF from human platelets was a gift of 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 [80]. Once established, the cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DME/F12) supplemented with 10% bovine calf serum (CS), and were subcultured within two 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 SV40 large T antigen (a gift of C. Reilly, Dept. of Pharmacology, Merck, Sharp, and Dohme Research Laboratories) were cultured as described [81]. 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 with 17% FCS. For C2C12 myoblasts, myotube formation was induced by incubating confluent cells 3-4 days in DMEM with 2% horse serum, whereas myoblasts were harvested for RNA isolation at 50% confluence. When cyclohexamide or actinomycin D was used, they were at concentrations of 20 μg/ml and 10 μg/ml, respectively.
*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 10-20% confluence, allowed to grow to >95% confluence (but not more dense than a monolayer), and then placed in low serum media containing 0.5% calf serum for three days. At this time, depending on the experiment, media was removed from the cells and replaced with fresh media containing fetal calf serum 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 media alone. Experiments were also performed in which PDGF was added to media without a media change. 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 one hour with 5 μCi/ml $^3$H-thymidine, after which trichloroacetic acid-precipitable counts were measured. In other experiments, sparsely plated cells growing in 20% FCS were placed in serum free media and RNA harvested at different time points for determination of Gax mRNA levels by Northern blot analysis.

*Northern blot analysis*

Total RNA from rat tissue and cultured cells was prepared using the guanidine thiocyanate method [82], fractionated on 1.2% agarose gels containing formaldehyde, and blotted onto nylon membranes. RNA from organs and cultured cells (Figure 7, A 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 [72] using a cDNA probe labeled by random priming and
consisting of a truncated Gax cDNA lacking the 5'-end and CAX repeat. Probes to 
Hox-1.3 and Hox-1.4 consisted of the cDNA's isolated from the rat aorta library and 
the probe to Hox-1.11 consisted of the Dral/EcoRI fragment of its cDNA. Blots were 
washed to a final stringency of 0.1-0.2X SSC, 0.1% SDS at 65°C. After all 
hybridizations 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 using 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, Gax levels 
were normalized to the corresponding GAPDH level determined on the same blot, to 
account for differences in RNA loading.

Results

The primary structure of Gax

To identify homeodomain proteins present in vascular smooth muscle, an adult 
rat aorta cDNA library [71] 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, thirteen positive clones were isolated, twelve of 
which contained homeodomains. Nine clones derived from previously described 
homeobox genes: Hox-1.3 [83], Hox-1.4 [84], Hox-1.11 [70, 85], and rat 
homeobox R1b [86]. Three clones represented the cDNA designated Gax.

The cDNA encoding Gax is 2244 base pairs 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 Gαx 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 [87] 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 (Figure 4). To confirm that this cDNA was capable of producing a protein product, the putative Gαx open reading frame was fused in frame to the pQE-9 E. coli expression vector (Qiagen, Inc., Chatsworth, CA) and expressed in bacteria [88]. 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 A/T-rich, MHOX-binding site [89] in the creatine kinase M enhancer (not shown).
Figure 4: The complete nucleotide and deduced amino acid sequence of Gax [74]. The predicted amino acid sequence is indicated below the nucleic acid sequence. The homeobox is indicated by a box, and the CAX repeat is underlined. A polyadenylation signal is in boldface and italics. Putative consensus sites for phosphorylation by protein kinase C are indicated by circles; for 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 [90].
Figure 5: Gax is homologous to Mox-1 [91] and more distantly related to the Dfd and proboscipedia homeobox genes [38]. A. Comparison of Gax with Mox-1. The homeodomains are indicated by boldface. Amino acid identities are indicated by “I” and conservative replacements by “+.” The numbering of Gax amino acid residues is indicated to the right of the sequence and the numbering of homologous Mox-1 segments are indicated below the segments. We also note that 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 “-” and consensus residues for all homeodomains are indicated by boldface. The three homeodomain putative α-helices are indicated by boxes.
The cDNA also contains a long 3'-untranslated region, from base 1109 to 2244 with a polyadenylation signal at base 2237 (Figure 4). The region between amino acids 87 to 184 is rich in serine (23/88 amino acids) and proline (10/88 amino acids), and contains several consensus sequences for phosphorylation by protein kinases [90]. *Gax* also possesses a feature found in several transcription factors, including homeodomain proteins, known as the CAX or *opa* transcribed repeat [92]. This repeat encodes a stretch of glutamines and histidines and in the case of *Gax* encodes eighteen residues, of which twelve are consecutive histidines. This motif is shared by other transcription factors, such as the zinc finger gene *YY1* [93, 94], as well as by several homeobox genes, including *H2.0, HB24, ERA-1 (Hox-1.6), Dual bar*, and *Tes-1* [52, 95-98]. 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 *anennapeda*, with only 54% amino acid identity [38]. 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 [91]. 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 (Figure 5A). At the nucleotide level, the homeodomains are 78% identical. Moreover, the 28-amino acid sequence immediately upstream and the three amino acids immediately downstream to 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 whose homeodomain sequence was recently reported [91].

*Gax* is less homologous to members of recognized classes of homeodomains [38]. It is similar to the *Drosophila* homeotic gene *proboscipedia* [99], which is identical to the *Gax* homeodomain in 40/61 amino acid residues (66% identity), *Hox-1.11*, with 39/61 (64%) amino acid identity [70, 85], and the recently reported *C. viridissima* gene *Cnax2*, with 37/61 (61%) amino acid identity [100]. (Figure 5B) The *Gax* homeodomain also shares homology with the homeodomains of several genes belonging to the class represented by the *Drosophila* gene *deformed* (*Dfd*), including: *Dfd*, 37/61 (61%) amino acid identity, *Hox-2.6*, 36/61 (59%) identity, *X. laevis Hox1A*, 34/61 (56%) identity, and *Hox-1.4*, 37/61 (61%) identity [38]. (See Figure 5B.) 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 [101]. Interestingly, the *Gax* homeodomain shares relatively little homology (26/61, or 43%, amino acid identity) with that of *MHo1*, the muscle-specific homeobox recently isolated by its ability to bind to an A/T-rich site in the creatine kinase enhancer [89], whose human homologue (*Phox*) has been shown to enhance the binding of the serum response factor to the serum response element [51]. With such a lack of homology, it is unlikely that *Gax* shares any function with *MHo1Phox*, 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* [91]. 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 x Mus spretus*)F1 X *C57BL/6J* mice [74]. This interspecific backcross mapping panel has been typed for over 1100 loci that are well distributed among all the autosomes as well as the X chromosome [75]. *C57BL/6J* and *M. spretus* DNA's were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLP’s) using a rat cDNA *Gax* probe. The 3.6 and 2.7 kb *M. spretus* HincII RFLP’s (see Materials and Methods) were used to follow 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, D12Nyul*, and *Spnb-1*. Although 161 mice were analyzed for every marker and are shown in the segregation analysis (Figure 6), 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: centromere- *Nmyc-1 - 19/193 - Lamb-1 - 9/166 - Gax - 10/166 - D12Nyul - 19/185 - Spnb-1*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± the standard error] are: *Nmyc-1 - 9.8 ± 2.2 - Lamb-1 - 5.4 ± 1.8 - Gax - 6.0 ± 1.9 - D12Nyul - 10.3 ± 2.2 - Spnb-1*. 
Figure 6: Gax maps in the proximal region of mouse chromosome 12. Gax was placed on mouse chromosome 12 by interspecific backcross analysis [74]. 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 x M. spreus)F1 parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of a M. spreus 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 GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).
The mapping studies demonstrate that \textit{Gax} is not a part of the HOX-1, HOX-2, HOX-3, or HOX-4 gene clusters, which are located on chromosomes 6, 11, 15, and 2, respectively [39, 102], nor does it cosegregate with any other homeobox genes previously mapped in the interspecific backcross. \textit{Mox-1} has been reported to reside on chromosome 11, and thus \textit{Gax} and \textit{Mox-1} do not form a novel homeobox gene cluster, although \textit{Mox-2} has also been mapped to chromosome 12 [91]. Based on 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 [38, 39]. 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 database maintained at The Jackson Laboratory, Bar Harbor, ME). \textit{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).
Figure 7: Expression of Gax in adult rat tissues by Northern blot hybridizations. 30 µg/lane of total RNA isolated from was separated on agarose gels containing formaldehyde, blotted to nylon membrane, and hybridized to a Gax probe as described in Materials and Methods, except for aorta, where only 20 µg were used. Each panel represents a separate experiment. A and B represent 30 cm gels, done to determine the size of the Gax transcript in various tissues and cultured cells, and C represents a 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. A= aorta, B= brain, H= heart, K= kidney, L= liver, Lu= lung, Sk= skeletal muscle, St= stomach, T= testes. Exposure times were one week for the Gax probe and 12 hours for the GAPDH probe. B and C. Distribution of Gax in cultured cells. Me= mesangial cells and HF= human foreskin fibroblasts. In B, exposure times were one week for the Gax probe and overnight for the GAPDH probe; in C, four days for Gax and 6 hours for GAPDH.
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 other tissues, and the heart (Figure 7A). Expression was also detected in adult lung and kidney. No expression was detected in brain, liver, skeletal muscle, spleen, stomach, or testes (Figure 7A), nor was expression detected in 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 (see Chapter 4).

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 - 2.4 kb Gax transcript was detected in smooth muscle cells cultured from adult rat aorta (Figure 7B). This is 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 SV40 (not shown) [81]. However, no expression was detected in either of two cell lines derived from embryonic rat aortic smooth muscle, A7r5 and A10 (Figure 7B). Gax was also not detected in NIH3T3 fibroblasts (Figure 7B), human foreskin fibroblasts (Figure 7C), or in C2C12 myoblasts or myotubes (data not shown). A relatively high level of Gax expression was detected in cultured rat mesangial cells (Figure 7C). Mesangial cells share many similarities to vascular smooth muscle, both structurally and functionally, and proliferate abnormally in renal diseases such as glomerulonephritis and glomerulosclerosis [103].
Figure 8: Time course of the down-regulation of Gax message during the G0/G1 transition. Quiescent rat VSMCs were stimulated with mitogen and the cells harvested at various time points for RNA isolation as described in the Materials and Methods section. 25 µg total RNA per lane were 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 to 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 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 hr. lane is slightly overloaded. 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 section). Key for graphs: PDGF-AA, open circles; PDGF-AB, open triangles; PDGF-BB, open squares; fetal calf serum, open diamonds.
**Gax is rapidly and transiently down-regulated during the G₀ to G₁ transition**

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 fetal calf serum or PDGF, potent mitogens for these cells (Figure 8). The down-regulation ranged from five- to nearly twenty-fold, depending on the mitogen used and the experiment, typically occurred within two hours after stimulation with serum or PDGF, and was maximal at 4 hours. The magnitude of down-regulation also tended to vary with the individual cell preparation. *Gax* transcript levels began to recover significantly by approximately 24 hours and approached baseline between 24-48 hours after stimulation. This rate of recovery tended to vary with the magnitude of the initial down-regulation and also with the individual rat VSMC preparations. A typical experiment is shown in Figure 8A, using PDGF isolated from human platelets as the mitogen, and a longer time course experiment is shown in Figure 8B, using fetal calf serum as the mitogenic stimulus. Rat VSMCs immortalized with the SV40 large T antigen retain many characteristics of VSMCs, including growth inhibition by heparin [81], and also down-regulate *Gax* in response to FCS in a manner identical to normal rat VSMCs (data not shown).
Figure 9: Time course of VSMC entry into S-phase as measured by $^3$H-thymidine uptake after mitogen stimulation. Quiescent cells were stimulated with mitogen, pulsed with $^3$H-thymidine for one hour at various time points and acid-precipitable counts measured as described in Materials and Methods. Each value for $^3$H-thymidine uptake represents the mean of four wells. Key for graphs: PDGF-AA, open circles; PDGF-AB, open triangles; PDGF-BB, open squares; fetal calf serum, open diamonds; no mitogen, open squares with cross.

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 [104], and they have differing potencies for stimulating DNA synthesis and inducing contraction in rat VSMCs [105, 106]. PDGF-AA is a relatively weak mitogen in VSMCs, whereas 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 isoforms (both at 10 ng/ml) or 10% fetal calf serum reduced Gax expression approximately 10-fold by 4 hours (Figure 8C). Qualitatively the effects were the same with these mitogens, although quantitatively the magnitude of the down-regulation was greatest with the fetal calf serum followed by 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 (Figure 8A). 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 if 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 fetal calf serum in their mitogenic potencies, as measured by their ability to stimulate 3H-thymidine uptake (Figure 9). PDGF-AA at 10 ng/ml, which was ineffective in causing Gax down-regulation (Figure 8C), stimulated DNA synthesis only weakly (Figure 9 and not shown). The PDGF-AB and -BB isoforms both stimulated cell proliferation as measured by 3H-thymidine uptakes at 15 hours, but fetal calf serum was the most effective mitogen. Dose-response experiments revealed that the ED50 for Gax down-regulation four hours after mitogen stimulation is between 4-8 ng/ml and 2-5 ng/ml for PDGF-AB and -BB, respectively (Figure 10A). Furthermore, a full mitogenic dose of 10% fetal calf serum suppresses Gax levels nearly 20-fold at four hours (Figure 10B), a larger effect than a maximal stimulatory dose of PDGF-BB (30 ng/ml), which has a 10-fold effect, or of PDGF-
AB, which has less than an 8-fold effect (Figure 10A). 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.

**Figure 10:** Dose-response curves for PDGF and serum in causing the down-regulation of \( Gax \). Quiescent VSMCs were stimulated with either PDGF-AB, -BB, or FCS at differing doses and the effect on \( Gax \) mRNA levels measured at four hours after mitogen stimulation, as described in Materials and Methods. A. Dose-response curve for PDGF-AB and -BB. Key: PDGF-AB, open triangles; PDGF-BB open squares. B. Dose-response curve for FCS. Curves represent the means of two to three experiments.
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% fetal calf serum caused a 40% decrease in Gax mRNA levels after four hours (Figure 10B), even though such stimulation increased \(^{3}\)H-thymidine uptake less than two-fold 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 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.

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 12-16 hours and 8-10 hours after mitogen stimulation, respectively [105, 107]. The recovery of Gax message levels appears to begin sometime in late S-phase or in G2, as Gax is detectable at 24 hours (Figure 8B). Gax message levels return to nearly normal approximately 48 hours after the initial stimulation, perhaps due to density-dependent growth inhibition. Similar behavior has been noted for gas1 and gas5, whose transcripts begin to reappear approximately 10-18 hours after serum stimulation [22, 29]. Finally, the expression of Gax is induced five-fold in VSMCs within 24 hours after rapidly growing, sparsely plated cells are placed in serum-free media (Figure 11). This time course is similar to the induction of gas genes in fibroblasts in response to serum starvation [22, 28, 29]. Thus, expression of Gax is regulated by the growth state of the cell, and its down-regulation is a prominent feature of the G0/G1 transition in these cells.
Figure 11: Induction of Gax expression in response to serum deprivation. Sparsely-plated VSMCs growing in media containing 20% FCS were placed in serum-free media, and RNA was harvested at various time points for Northern blot analysis and subsequent quantification of Gax mRNA levels as described in Materials and Methods. Values for Gax message level were normalized to the intensity of the GAPDH signal.

*Gax down-regulation is most likely not due to mRNA destabilization*

To begin to examine whether the mechanism of Gax down-regulation is transcriptional or post-transcriptional, we measured Gax mRNA levels by Northern blot analysis in quiescent and mitogen-stimulated cells in the presence of actinomycin D, quantifying the mRNA level by exposing the blot to the PhosphorImager and integrating the volume pixel values under each band as described in the Experimental Methods section. For comparison, and in order to control for differences in loading which might not be apparent by ethidium bromide staining of the gels prior to blotting,
we reprobed the Northern blots with a probe for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH transcript is generally very stable, with a half-life in thyroid cells of between 9 and 56 hrs., depending upon the whether the cell has been stimulated with thyrotropin or not [108]. Its half-life in VSMCs has not been reported. In our experiments, we could detect no significant difference between GAPDH mRNA levels at T=0 and T=6 hours in VSMCs treated with the transcriptional inhibitor actinomycin D, either in the presence or absence of serum, implying a long half-life for GAPDH in these cells as well (data not shown). Therefore, we normalized our Gax levels to the corresponding GAPDH level to adjust for any uneven loading between lanes and provide more accurate quantification of Gax mRNA levels.

In quiescent VSMCs, the Gax transcript is relatively labile, with an estimated half-life of 1.7 hrs. This is relatively short compared to the half-lives of the clustered homeobox mRNAs, which were estimated to be between 3-4 hours in NT2/D1 cells [109]. In both quiescent cells and those stimulated with mitogen there was a small and variable but reproducible increase in Gax mRNA levels of 0-50% one hour after the addition of actinomycin D, after which Gax mRNA decayed exponentially with an estimated half-life of 1.7 hours (Figure 12). The fits of the resulting curves to a single exponential equation were excellent, with \( r^2 = 0.99 \) and 0.95 for the quiescent and PDGF-stimulated cells, respectively. The same results were obtained when cells were stimulated with fetal calf serum (data not shown); i.e., within experimental error, there was no significant difference detectable in the Gax mRNA half-life in quiescent or stimulated cells, whether the mitogen was serum or PDGF. Based on these experiments, we cannot rule out a small effect of mitogen on Gax mRNA stability, but there is no dramatic effect. The estimated half-lives for the transcripts in quiescent cells and mitogen-stimulated cells varied by less than five minutes, well within experimental
Figure 12: Effect of RNA synthesis inhibitors on Gax downregulation in response to mitogens. VSMCs made quiescent by incubation in low serum medium (see Materials and Methods) were pretreated with actinomycin D for 30 min. prior to mitogen stimulation. One experimental group (open squares) served as a control and was not stimulated with mitogen. A second experimental group (open diamonds) was treated with PDGF-BB, 20 ng/ml. mRNA was harvested at the indicated time points and Gax mRNA levels were measured from band intensities on the PhosphorImager and normalized to the band intensity of GAPDH, as described in Materials and Methods. Each point represents the mean of three experiments. The data is plotted on a logarithmic scale to show the that the decline in the Gax mRNA level is exponential after its peak.

error. Based on this experiment, we tentatively conclude that a change in RNA stability alone is insufficient to account for the rapid decline in Gax mRNA levels upon mitogen stimulation.

The reason for the brief induction of the Gax mRNA by actinomycin D is unclear. It is possible that it may represent a direct effect of actinomycin D on the cells.
An alternative explanation is that the inhibitor is preventing the transcription of an mRNA coding for a labile protein that accelerates the breakdown of the Gax mRNA, allowing it to accumulate briefly. During the course of these experiments, it became apparent that the overall time course of the decline of Gax mRNA due to mitogen was somewhat slower in the presence of actinomycin D than when actinomycin D was not present. (Compare Figure 8C with Figure 12.) If the decline of Gax mRNA after mitogen stimulation in the absence of RNA synthesis inhibitors is examined, it is seen that by four hours Gax mRNA levels have fallen by 10-fold, whereas in the presence of actinomycin D, Gax mRNA levels fell only 5-fold four hours after the peak of its brief induction. If we assume that the transcription of the Gax mRNA is terminated upon mitogen-stimulation, then, based on its half-life as estimated by treatment with transcriptional inhibitors, we would expect the level of Gax transcript to have fallen only by 5.1-fold. The 10-fold decline after four hours is more consistent with a half-life of 1.2 hours, assuming transcription is completely halted, and an even shorter half-life, if a low level of transcription continues. Although this could possibly be within experimental error, given the difficulties in determining the exact level of an mRNA as scarce as Gax, the discrepancy is rather large to ignore. One possible explanation is that the down-regulation of Gax mRNA, at least in part, requires the synthesis of a labile protein, which is inhibited by transcriptional arrest induced by actinomycin D. One indication that this might be the case is that the down-regulation by serum and PDGF is partially blocked by treatment with the protein synthesis inhibitor cyclohexamide, which attenuates the maximal down-regulation by approximately 50-75% (Figure 13). Thus the PDGF-induced down-regulation of Gax seems to depend, at least in part, on new protein (and therefore presumably RNA) synthesis. The full elucidation of whether the mechanism of Gax down-regulation is transcriptional will
Figure 13: Effect of protein synthesis inhibition on the mitogen-induced down-regulation of the Gax transcript. Quiescent cells were induced with 10% FBS as described in the Materials and Methods section. One experimental group was pretreated with cyclohexamide 20 μg/ml for 30 min. and the same concentration of cyclohexamide was maintained in the media throughout the experiment (open diamonds) and one group was not treated with cyclohexamide and served as a control (closed squares). Total RNA was isolated from cells at the indicated time points and subjected to Northern blot analysis with the Gax cDNA probe. Band intensities were quantified on the PhosphorImager and normalized to GAPDH band intensities as described in Materials and Methods.

require nuclear run-on experiments and an analysis of the Gax promoter, projects which are presently ongoing in our laboratory.
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, migration, and proliferation. Evidence has also been accumulating that the deregulation of homeobox gene expression can lead to uncontrolled cell growth and disease [40, 47, 48, 56, 63, 110]. 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 back-cross analyses. In the adult rat, its transcript can only be detected in 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 [10, 11, 13, 17, 111-113]. Evidence for Gax expression in vascular myocytes include the isolation of this cDNA from an adult aorta library, the detection of Gax transcripts in adult aorta by Northern blot (Figure 7A) and in situ hybridization (not shown), as well as the detection of Gax expression by Northern blot in vascular myocytes that were cultured from adult rat aorta (Figure 7B) and in SV40-transformed smooth muscle cells (not shown). The Gax mRNA is also detected in whole kidney and in mesangial cells cultured from this organ (Figure 7C). 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 [103].

Vascular myocytes differ from skeletal and cardiac myocytes in that they are capable of re-entering 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, fetal calf serum, produced the largest down-regulation, and the recovery of $Gax$ to pre-stimulus 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 different intracellular signaling pathways than the AB or BB isoforms [105, 106], which may explain its relative 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 [7, 107], and these cells enter S-phase 10 to 16 hours after stimulation [105]. (Also, see Figure 9.) 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 [114]. Since the down-regulation of $Gax$ significantly precedes the up-regulation of $c$-myb [107], the induction of DNA synthesis [105], and the down-regulation of smooth muscle contractile proteins [2], 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 known to date to be 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 (bFGF) and members of the transforming growth factor-β family have been shown to regulate homeobox gene expression in developing *Xenopus* embryos [115, 116].

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 [96]. 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 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 hours and maximal by 4 hours, the up-regulation of other homeobox genes by bFGF or interleukin-2, takes many more hours [96, 115, 116], and, in the case of the differentiation agent retinoic acid, can take days [109]. 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 is accumulating that homeobox genes are indeed directly involved in growth control in many cell types. There are now examples of homeobox genes that can act as oncogenes [40, 47, 48, 56, 63, 110]. 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 [40]; overexpression of *Hox-7.7* in myoblasts inhibits terminal differentiation and causes cell transformation [63], and overexpression in T-cells is similarly oncogenic [48]. 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 [117]. Evidence of the ability of homeobox genes to regulate the activity of immediate early gene products also comes from the recently described human gene (whose mouse homologue 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 [51]. Whether or not *Gax* interacts with immediate early genes remains to be proven; however, given its rapid down-regulation when quiescent cells re-enter 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 G0/G1 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 NIH3T3 fibroblasts [22-24, 28, 29]. 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 [24, 28, 30, 31], and one (*gas2*) encodes a protein which has been shown to be a component of the microfilament system [118]. One of the gadd genes, gadd45, has been implicated in a cell cycle checkpoint control pathway involving its activation by p53 [33]. 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 (K. Guo and K. W., unpublished result). As an example of this potential, one gadd gene, gadd 153, is a C/EBP-related gene whose expression is induced by growth arrest signals and DNA-damaging agents such as nephrotoxic cysteine
conjugates [20, 21, 25-27]. Likely representing the hamster homologue of CHOP-10 [32], its mechanism of action appears to be to dimerize with other C/EBP-related proteins through its leucine zipper motif and prevent them from functioning as transcriptional activators. Future studies will test whether Gax has a direct growth repression function like gas1 [24], 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.

**Genbank Accession Number**

The nucleotide sequence of Gax has been submitted to the Genbank™ and EMBL databases under the accession number Z17223.

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In Chapter 6, we present experimental evidence that Gax functions to inhibit VSMC proliferation in response to mitogens.


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CHAPTER 4:

GAX EXPRESSION DURING EMBRYOGENESIS

Introduction

Homeobox genes are a class of transcription factors long known to be important in cell differentiation and growth during embryogenesis [1-17]. The proteins encoded by these genes are transcription factors with a helix-turn-helix motif that bind to A/T-rich consensus sequences with high affinity [4, 16, 18]. 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 [10, 14, 19]. 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 [1-3, 8-11, 13-17, 20-26]. Examples of this regulation are found in the pituitary (GHF-1/pit-1) [1, 8], the immune system (oct-2) [25], pancreas (isl-1) [23], and liver (HNF-1) [21], among others. Further evidence for the importance of these genes in growth regulation comes from the observation that they can, when overexpressed in cells, be oncogenic [17, 20, 22, 24, 27, 28].

In spite of this involvement in differentiation and growth in a wide variety of tissues, little is known about the involvement of specific homeobox genes in the cardiovascular system. Two homeobox genes have been implicated in cardiovascular development in vertebrates. The first is Hox-1.5, whose importance in the
cardiovascular system during embryogenesis was demonstrated when transgenic mice homozygous for a null mutation in this gene were shown to have severe anomalies of the heart and great vessels that were incompatible with postnatal life, as well as many branchial anomalies, the overall constellation of which resembled the human disease DiGeorge’s syndrome [2]. The second, Hox-1.11 has been isolated from a vascular smooth muscle cDNA library, and its expression during embryogenesis in the cardiovascular system hints that it may be involved in the changes undergone by the cardiovascular system at birth, when its expression ceases in the heart, but continues in the great vessels [29]. Recently, however, a homeobox gene, Gax, has been isolated from a rat vascular smooth muscle cDNA library [5]. Gax is unique in that its expression is rapidly down-regulated in vascular smooth muscle cells (VSMCs) during the G0/G1 transition when quiescent cells are stimulated to divide by mitogens [5].

Vascular smooth muscle cells (VSMCs) have an important role in the pathogenesis of atherosclerosis and coronary restenosis [30, 31]. Unlike the case for skeletal muscle [32-36], little is known about the developmental molecular biology of this cell type, although more is known about its developmental biology [37-43]. Based on its tissue-specific expression and its unique regulation according to the proliferative state of the cell, we considered it likely that Gax also has a significant role in the development of the vasculature, and therefore we determined its pattern of expression during embryogenesis in the mouse. Gax is widely expressed throughout embryogenesis in tissues of ectodermal and mesodermal origin, including the neural tube, the somites (which give rise to skeletal muscle), limb buds, and branchial arches 3 and 4, as well as the region of the heart prominence. Its expression becomes more restricted as development proceeds, and in neonates its expression is largely confined to the cardiovascular system. Thus it is likely that Gax, in addition to a potential role as
a growth inhibitory gene in VSMCs. may also have an important role in the
development of the cardiovascular system.

**Materials and Methods**

*In situ* hybridizations were carried out on developing mouse embryos and
selected rat tissues as previously described [44], using a riboprobe generated by
transcribing the antisense strand of the *Gax* cDNA from nucleotide positions 954 to
1154 with α-35S-UTP [5]. This probe lacked the homeodomain and CAX repeat, but
contained the C-terminal end of the coding region and a short length of the 3'-
untranslated region, which share no homology to *Max-1*, a homeodomain gene whose
homeodomain is identical to that of *Gax* at the peptide level [45]. Histological sections
were incubated with 0.5 X 10^6 cpm of probe for 16 hours at 50°C in 50% formamide,
and 4X SSC. Tissue sections were then sequentially washed in 5X SSC, 10 mM
dithiothreitol for 30 minutes at 50°C; 50% formamide, 2X SSC, and 0.1 M
dithiothreitol for 20 minutes at 65°C; treated with 20 µg/ml RNase A for 30 min. at 37°C
and then washed for 15 minutes each in 2X SSC and 0.1X SSC at 37°C. The
sections were then dehydrated by sequential washes in solutions with increasing
concentrations of ethanol and dried. Autoradiography was performed with Kodak
NTB-2 photographic emulsion, and the sections were developed, fixed, and stained in
0.2% toluidine blue after 10 days. Sections were examined under light and dark field
microscopy. Density of silver grains in relation to the background and confirmation of
intracellular location of grains was determined under high power objectives. As a
control, identical sections were hybridized with a probe made from a different
homeodomain cDNA (*Hox-I.11*), which produced a different pattern of hybridization
[29].
Figure 14: Expression of Gax during mid-development as determined by in situ hybridizations. (See this page and the next page.) Mouse embryos of the indicated age post coitum were sectioned, fixed, and hybridized with a Gax cRNA probe as in Materials and Methods. A (dark field): Coronal section of the whole embryo at 9.75 days, with Gax expression seen in the neural tube, the third and fourth aortic arches, the third and fourth branchial arches, and the limb buds. B (dark field) and C (bright field): Sagittal section of a whole embryo at 10.5 days p.c., 40X magnification. At this time, expression is observed in the anterior neural tube to approximately rhombomere 2, in the third and fourth visceral arches, and the limb bud. Key for Figures 14-18: b = branchial arch 1; dor = dorsal; e = eye; lb = limb bud bud; n = neural tube; r = rib; s = somite; sc = sclera; v = vibrissa.
Results

The expression pattern of Gax was analyzed by in situ hybridization of mouse embryo sections (Figures 14-18). The pattern of Gax expression in the adult has already been described in Chapter 3 [5]. Using a 201-nucleotide cRNA probe lacking the homeodomain and CAX repeat but containing the C-terminal peptide coding region, which shares no homology with Mox-I [45], we determined that during embryogenesis Gax expression is not lineage-restricted and that the transcript is detected in tissues derived both from ectoderm and mesoderm, but not in tissues derived from endoderm.

In the central nervous system Gax transcripts are detected during mid-embryogenesis in the ventral portion of the neural tube with its rostral boundary in the hindbrain and myelencephalon, at or just caudal to the first rhombomere (Figure 14, C and D). Whereas the rostral boundary of Gax expression is very sharply demarcated, the caudal boundary is diffuse, with labeling being most intense towards its rostral boundary of expression and gradually decreasing caudally, but still detectable near the caudal neuropore. This is in contrast to another gene isolated from the same vascular cDNA library, Hox-I.11, whose expression is more uniform and does not taper off as markedly in the caudal portion of the neural tube [29]. By day 15, Gax expression in the central nervous system is greatly diminished (not shown) and Gax is not detectable in the adult brain by Northern blot (Chapter 3, Figure 7).

Another ectodermal tissue in which Gax is also expressed is the eye. In early to mid-embryogenesis, Gax is not detectable in the optic vesicle or in any of the structures around it (Figure 14, B and C); however, at day 15, it is expressed in the developing lens, as well as in the pigmented layer of the eye (Figure 15). Msh-like homeobox genes (Hox-7.1 and Hox-8.1) are known to participate in pattern formation in the eye [15], and Gax also appears to be involved in this process. Strong expression is also
seen in the primordial dermal root sheath surrounding the developing whiskers in later embryogenesis, as well as the outer root sheath, which is immediately adjacent to the dermal root sheath and derived from ectoderm (Figure 16, A and B).

*Gax* transcript was also detected in many tissues derived from mesoderm. In early to mid-embryogenesis, transcript was detected in the visceral arches, and was especially strong in the region of the heart prominence, but was not expressed in the mandibular and hyoid arches (Figure 14, B and C). Expression was also observed in the somites (Figure 17, A and B), which give rise to skeletal muscle and connective tissue, and in the limb buds (Figures 14 and 16). By day 15, *Gax* expression is activated at very high levels in skeletal muscle (Figure 17C), and may be associated with the differentiation of the mesenchyme of the somites into myoblasts, which occurs around day 13. Of note, however, *Gax* expression in skeletal muscle ceases before birth (not shown) and remains off in the adult (Figure 5A, Chapter 3). Overall, *Gax* expression is much more widespread in tissues of mesodermal origin than in those of ectodermal origin, and the pattern of expression, as determined by Northern blot analysis of RNA from adult tissues, was consistent with the pattern determined in later embryos by *in situ* hybridizations.
Figure 15: Sagittal section of mouse eye, 15 days p. c., 100X magnification, dark field. *Gax* is expressed in the lens and the pigmented layer of the retina, as well as in the extraocular muscles. Key for Figures 14-18: b = branchial arch 1; dor = dorsal; e = eye; L = lumen; lb = limb bud; n = neural tube; r = rib; s = somite; sc = sclera; v = vibrissa.
Figure 16: Expression of *Gax* during late development as determined by *in situ* hybridizations. A (dark field) and B (bright field): Coronal section of snout and forelimb, 15 days p. c., 40 X magnification. *Gax* is expressed in the developing limb, the outer root sheath of the vibrissae (whiskers), which is derived from ectoderm, and the dermal root sheath, which is derived from mesoderm. Key for Figures 14-18: b= branchial arch 1; dor= dorsal; e= eye; lb= limb bud; L = lumen; n= neural tube; r= rib; s= somite; sc= sclera; v= vibrissa.
Throughout embryogenesis and adulthood, *Gax* expression is prominent in the cardiovascular system. Expression was detected in many developing arteries and veins by mid-embryogenesis, days 9-10, and its expression continued throughout development and into adulthood. *Gax* transcript was detected in the third and fourth aortic arches but not the first and second (Figure 14), which may be significant because the third and fourth arch give rise to the common carotid arteries and the aortic arch, respectively, whereas the first and second arches largely disappear, during later embryogenesis [46]. In most regions of the embryo, smooth muscle is induced to form in the surrounding mesenchyme by hemangioblasts, the precursors of vascular endothelial cells, by mechanisms of angiogenesis which continue to be used throughout development and in the adult organism in the repair of injury [43]. In this region, the mesenchyme is derived from cells migrating from the neural crest. *In situ* hybridization analyses detected *Gax* transcripts in the ascending aorta of neonates (Figure 18A) and adults (not shown), and during embryogenesis expression was detected in many arteries and veins, including the aortic arches, the cardinal vein, and the developing pulmonary artery and inferior vena cava (Figure 18B). The *Gax* transcript was readily detectable by Northern blot hybridizations in adult rat aorta at levels higher than in other adult tissues (Chapter 3, Figure 7A).

Expression in the heart was also detected at day 15, when weak expression is first seen in the ventricular cardiomyocytes (not shown). Unlike the case in skeletal muscle, however, *Gax* continues to be expressed in myocardial cells throughout development and can be detected in neonatal and adult heart by *in situ* hybridization (not shown) and in adult heart by Northern blot hybridization (Figure 7A, Chapter 3). In later development, transcript is also detected in the kidney, but appears to be primarily confined to the vasculature of the renal pelvis (Figure 5A and data not shown). As
shown in Chapter 3, strong Gax expression is also detected in mesangial cells cultured from rat kidney. Finally, Gax is expressed in fetal and adult lung. In the developing embryo, transcript is detected in the mesenchymal interstitial cells during the late canalicular period, but is largely excluded from the cuboidal epithelial cells of the developing alveoli (not shown). We note that many smooth muscle cell markers have been identified in the developing lung, including smooth muscle α-actin, desmin, and smooth muscle myosin [47, 48].
Figure 17: Gax is expressed in somites and transiently in skeletal muscle. (See this page and the next page.) A and B: Parasagittal sections through the developing somites of an 11.5 day p. c. embryo, 100X magnification, dark field. Gax is expressed at moderately high levels in the somites, which ultimately develop into skeletal muscle and bone. C. Parasagittal section through a 15 day p. c. embryo at the level of the thoracic segments and rib cage, 40X magnification, dark field. Gax is expressed at high levels in developing skeletal muscle at this stage, but this expression ceases shortly before birth and remains off though adulthood. Elsewhere in the same section, no Gax expression was observed in liver, or intestine. Key for Figures 14-18: b = branchial arch 1; dor = dorsal; e = eye; lb = limb bud; L = lumen; n = neural tube; r = rib; s = somite; sc = sclera; v = vibrissa.
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 [1-3, 8-17, 20-26, 49-52]. Evidence has also been accumulating that the deregulation of homeobox gene expression can lead to uncontrolled cell growth and disease [17, 20, 22, 24, 27, 28]. In this study we have determined the expression of a cardiovascular, growth arrest-specific homeobox gene, Gax, during embryogenesis. Gax is expressed in a wide range of tissues in the developing embryo including those derived from mesoderm and ectoderm, but not endoderm. As development proceeds, the expression pattern of Gax becomes progressively more restricted, and in the adult transcripts can only be detected in aorta, heart, kidney and lung. Expression is detected in the cardiovascular system throughout development, but expression in other organs and in ectodermally derived tissues ceases around the time of birth. 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 (Chapter 3), the detection of Gax transcripts in adult aorta by Northern blot and in situ hybridization (Figure 18), and the detection of Gax expression in vascular myocytes that were cultured from adult rat aorta (Chapter 3). Gax expression detected in the kidney appears to be confined to the vasculature of the renal pelvis (not shown) and a signal is also detected in mesangial cells cultured from this organ (Chapter 3). 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
critical to the pathogenesis of various forms of glomerulosclerosis and glomerulonephritis [53].

The pattern of Gax expression during development does not appear to follow the same pattern as the expression of members of the HOX-1 through HOX-4 clusters in that its boundaries of expression do not closely follow the body axis [7, 14, 19]. Rather, its boundaries tend to follow a more proximal-distant gradient relative to the main body axis rather than a rostral-caudal gradient. This pattern of expression is more like that of Hox-7.1 [44], a gene known to be important in pattern formation in the vertebrate eye [15], as well as recently having been shown to be an oncogene [17]. Moreover, unlike the recently reported genes, Mox-1 and Mox-2, both of which share identical or near-identical homeodomains with Gax and whose expression is reported to be restricted to tissues of mesodermal origin during embryogenesis [45], Gax is expressed in several tissues of ectodermal origin at different times during embryogenesis, including the central nervous system and eye. We could not, however, detect the Gax transcript in any tissues of endodermal origin at any time during development or in the adult.
Figure 18: Gax expression in blood vessels. A. Cross-section of neonatal rat aorta. 100X magnification, dark field. Gax transcript is detected in the smooth muscle of the arterial wall. B. Longitudinal section of inferior vena cava. 15 days p.c.. Key for Figures 14-18: b = branchial arch 1; dor = dorsal; e = eye; lb = limb bud; L = lumen; n = neural tube; r = rib; s = somite; sc = sclera; v = vibrissa.
In terms of muscle development, *Gax* appears to play a role in the development of all three kinds of muscle. First, in cardiac muscle, *Gax* is expressed at least from mid-embryogenesis and continues to be expressed constitutively throughout development and into adulthood. In skeletal muscle, it is expressed transiently between days 13 and 16, which is the stage when the mesenchyme of the somites begins to differentiate into myoblasts, implying that *Gax* may play a facilitatory role in this process. Finally, in vascular myocytes *Gax* is expressed from mid-development, just as it is in cardiac muscle. This is not surprising, given that the heart forms from paired blood vessels, and thus the heart and major vessels develop from different parts of the same embryonic precursor [46]. This expression continues into the adult organism, where the only organs in which *Gax* is expressed are the heart, arteries, lungs, and kidneys [5], all of which are either part of the cardiovascular system or are themselves highly vascularized. Presumably, in other organs in which *Gax* is expressed during embryogenesis but in which its expression ceases prior to birth, *Gax* may play a role in development, but its role is such that it is only needed for a defined period of time.

With a complex pattern of expression like this, it is necessary to determine more precisely the role of Gax in development. The most direct way to accomplish this is to produce a transgenic mouse homozygous for a null mutation in *Gax* and determine its phenotype throughout development, as has been done for *Hox-1.5* [2]. Like the case of *Hox-1.5*, it is likely that there will be tissues for whose normal development Gax is necessary and those for which it is not. It is likely that, as was the case for *Hox-1.5-/-Hox-1.5-/-* homozygotes [2], the defects found in *Gax/Gax* transgenic mice will not simply correlate with the pattern of *Gax* gene expression in normal mouse embryos. For example, tissues that express *Hox-1.5* during embryogenesis yet show no defects in the homozygous mice include components of the nervous system, lung, stomach,
spleen and kidneys. Presumably, the lack of a mutant phenotype in these organs
results from functional redundancies between Hox-1.5 and other regulatory molecules.
Thus, it is not possible to predict with any confidence from the expression pattern of
Gax which tissues will be affected by loss of Gax function during development.
However, from the pattern of Gax expression in the cardiovascular system during
development and its unusual regulation in VSMCs, there is a reasonable likelihood that
Gax will ultimately prove to be very important in development and pattern formation in
the cardiovascular system.

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CHAPTER 5:

EXPRESSION OF A HOMEobox GENE (MHOX/PHOX) THAT IS RAPIDLY AND TRANSIENTLY INDUCED DURING THE G0/G1 TRANSITION IN VASCULAR SMOOTH MUSCLE CELLS

Introduction

In vascular smooth muscle cells (VSMCs), growth factors regulate both cell proliferation and the state of cell differentiation by binding to cell surface receptors, activating various intracellular signaling pathways and transmitting the proliferative signal to the nucleus, which responds by activating and repressing batteries of genes appropriate for the proliferative state [1-4]. Like skeletal and cardiac muscle cells, VSMCs in vivo are in a quiescent state. However, unlike other types of muscle cells, which are terminally differentiated, VSMCs retain the ability to respond to serum and platelet growth factors, re-entering the cell cycle in response to appropriate signals [1, 4, 5]. Moreover, upon becoming proliferative they dedifferentiate, inactivating tissue specific genes such as the one for smooth muscle α-actin, and activating genes that are not tissue-specific, such as those for the nonmuscle actins [6-12]. They are able to return to their quiescent, differentiated state when the inciting mitogenic stimulus is no longer present. Such processes are undoubtedly critical during normal development in determining the architecture of the vascular tree and in repairing arterial injury [1, 13, 14]. They also come into play during adulthood in certain disease states, such as atherosclerosis and coronary artery restenosis after balloon angioplasty, where the normal growth of these cells is disordered, resulting in excessive growth of these cells.
and leading to narrowing and occlusion of the lumen with resultant end organ hypoxia due to compromised perfusion [10-12].

Unlike the case in skeletal muscle [15, 16], the mechanisms underlying the regulation of VSMC differentiation and proliferation remain largely unknown at the nuclear level. Much effort has been devoted to characterizing the growth factors which act as VSMC mitogens [4, 5, 12, 17-21], the receptors which bind them [22-24], and the effects these mitogens produce in terms of signaling pathways and genes that are activated [6, 9, 10, 12, 25, 26]. Much less is known about potential final downstream targets of these pathways or what specific transcription factors might be responsible for translating the mitogenic signal into the activation of sets of proliferative genes and/or the repression of sets of growth arrest genes.

Homeobox genes encode a class of transcription factors important in cell differentiation and growth during embryogenesis, both in vertebrates and invertebrates; other processes in whose control homeobox genes have been implicated include pattern formation, tissue-specific gene expression, growth control, and oncogenesis [27-40]. Until recently, it was unknown if these genes play a role in VSMC growth, but now several genes from the HOX-1 (HOX-A) cluster have been isolated from VSMCs, as well as a diverged homeobox gene, Gax, which has the expression properties of a growth arrest-specific gene in VSMCs, with its expression being rapidly down-regulated when quiescent VSMCs are induced to proliferate with mitogens [41, 42].

Whatever tissue-specific transcription factors are involved in the control of VSMC proliferation, it is likely that at least some of them interact with ubiquitous factors, such as the serum response factor (SRF), which plays a critical role in the activation of mammalian genes by growth and differentiation factors [43]. Indeed, recently a homeobox gene (known as Phox or MHOx) was isolated by two independent approaches by two different groups: first, by complementation screening in yeast for
factors which can cooperate with MCM1, a yeast mating type gene containing a MADS box and sharing homology to the SRF, to activate cell type-specific gene expression [44]; and, second, by its ability to bind to an A/T-rich site essential for muscle specific transcription and trans-activation by myogenic helix-loop-helix proteins in the muscle creatine kinase enhancer [45]. *MHox/Phox* has been shown to be able to interact with the SRF and increase its DNA-binding affinity for the serum response element (SRE), thus increasing its activity [44].

Because of the apparent dual function of *MHox/Phox* participating in the regulation of expression by myogenic promoters in muscle and in the regulation of *c-fos* and immediate early gene expression by its interaction with SRF, and because recent work in our laboratory has identified a mitogen-responsive homeobox gene, we considered *MHox/Phox* to be another possible candidate for a homeobox gene that might play a regulatory role in the growth and differentiation of VSMCs. As a first step in determining its potential role, we demonstrated its expression in cultured VSMCs. We then investigated whether its expression is regulated by mitogenic signals, and found that, in marked contrast to the behavior of *Gax* [41], *MHox/Phox* is rapidly and transiently up-regulated in quiescent VSMCs when they are stimulated with serum or platelet growth factors. This up-regulation occurs with a time course similar to that of *c-myc* [2], making it only the second known mammalian homeobox gene whose expression level is strongly regulated by peptide growth factors. These observations are consistent with the postulated role of *MHox/Phox* interacting with the SRF to enhance its binding to the SRE and imply that it may have a positive regulatory role in VSMCs in the G0/G1 transition [44].
Materials and Methods

Reagents and cell culture

Recombinant platelet-derived growth factor A/A, A/B, and B/B were obtained from Boehringer-Mannheim, and PDGF from human platelets was a gift of 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 [46]. Briefly, thoracic aortas from young rats were isolated, and the endothelial and adventitial layers were carefully removed under sterile conditions. They were then dissociated in trypsin and elastase and plated in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 (DMEM/F12) with 10% bovine calf serum (CS). Once established, the cells were grown in DMEM/F12 and were subcultured at a 1:5 split within three days after reaching confluence. Cell preparations were labeled with monoclonal antibodies to smooth muscle α-actin (Sigma Chemical Co.) to verify identity (data not shown), and were between passages 4 and 10 when used for experiments. NIH-3T3 fibroblasts were obtained from American Type Culture Collection and cultured as recommended by the supplier in DMEM with 10% CS.

Mitogen inductions

Experiments in which rat aorta cells were stimulated with serum or PDGF were performed essentially as described [41]. Cells were plated at a density of 10-20% confluence, allowed to grow to >95% confluence (but not more dense than a monolayer), and then placed in media containing 0.5% calf serum for three days to induce quiescence. At this time, depending on the experiment, media was removed
from the cells and replaced with fresh media containing either fetal calf serum 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 media alone. Experiments were also performed in which PDGF was added to media without a media change. 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 MHox/Phox up-regulation experiments were stimulated with mitogen and pulsed at various time points after stimulation for one hour with 5 $\mu$Ci/ml $^3$H-thymidine, after which trichloroacetic acid-precipitable counts were measured.

*Northern blot analysis*

Total RNA from rat tissue and cultured cells was prepared using the guanidine thiocyanate method [47], fractionated on 1.2% agarose gels containing formaldehyde, and blotted onto nylon membranes. Hybridizations were carried out at 65$^\circ$C in a buffer consisting of 0.5 M sodium phosphate (pH 7.0), 7% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 1% bovine serum albumin [48]. Blots were washed to a final stringency of 0.1-0.2X SSC (1X SSC = 150 mM NaCl, 26 mM sodium citrate, pH 7.0) and 0.1% SDS at 65$^\circ$C. Probes were labeled to a specific activity of at least 1 x $10^9$ cpn/ug by random priming, and consisted of a cDNA to MHox (clone Q10) [45] or a cDNA to Gax missing approximately 500 bases from its 5' end [41]. After all probings with the homeobox probes were complete, blots were rehybridized with a probe to rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to demonstrate message integrity and to serve as an internal control for the amount of RNA loaded.

For experiments involving the measurement of MHox/Phox upregulation and Gax down-regulation by mitogens, MHox/Phox, Gax (where applicable), and GAPDH
mRNA levels for each lane were quantified by scanning laser densitometry of the appropriate autoradiogram. In all quantitative comparisons of *MHox/Phox* mRNA levels between experimental groups *MHox/Phox* levels were normalized to the corresponding GAPDH level determined on the same blot, to account for differences in RNA loading.

**Results**

Because of our interest in factors regulating the growth and differentiation of VSMCs, we tested whether *MHox/Phox* expression is regulated by growth factors in cultured VSMCs, after having first determined that it is expressed as a major transcript of 4.0 kb in these cells (Figure 19), whereas in most mesodermal cell types it is expressed as two major bands at 3.6 kb and 4.0 kb [45]. It was found that the *MHox/Phox* transcript is rapidly and transiently upregulated up to eight-fold within four hours after quiescent VSMCs are stimulated with 10% FBS, with levels returning to near normal within 8 hours (Figure 19).
Figure 19: Up-regulation of \(MHox\) transcript in VSMCs in response to mitogen stimulation. Quiescent VSMCs were stimulated with 10% FCS as described in the Materials and Methods section, and the cells harvested at various time points for RNA isolation by the guanidine thiocyanate method [47]. The RNA was then subjected to Northern blot analysis using probes to MHox, followed by a probe to GAPDH.
Because platelet-derived growth factor is an important mitogen for VSMCs [12, 49, 50], we examined whether Mlox/Phox could be upregulated in a similar fashion by this growth factor alone. PDGF consists of two chains, designated A and B, and consists of three isomers, PDGF-AA, -AB, and -BB [49]. PDGF-AA upregulated Mlox/Phox, but only weakly (Figure 20), whereas PDGF-BB produced an upregulation nearly as potent as FBS (Figure 21), as does PDGF derived from human platelets (Figure 22A). Moreover, the upregulation occurs in a time frame after mitogen stimulation very similar to the time frame in which the down-regulation of Gax, a recently described homeobox gene isolated from VSMCs whose pattern of expression resembles that of a growth arrest-specific (gas) gene [41], occurs (Figure 21). The maximal mitogen-stimulated level of Mlox/Phox expression is comparable to the level of expression in randomly cycling NIH3T3 cells (Figure 21). The relative potencies of different mitogens for Mlox/Phox upregulation, based on scanning densitometry of the autoradiograms, normalized for GAPDH expression is shown in Figure 22B.

Pretreatment of cells with cyclohexamide 20 μg/ml did not produce a superinduction of the Mlox transcript (data not shown).

Finally, the regulation of Mlox/Phox expression by mitogens was also examined in a continuous cell line (NIH3T3). Serum and PDGF-BB also upregulate Mlox/Phox expression in NIH3T3 cells with a magnitude similar to that observed in VSMCs, although the time course appears to be slightly earlier (Figure 23).
Figure 20: Effect of PDGF-AA on Mhox expression in VSMCs. Quiescent VSMCs were stimulated with 10 ng/ml recombinant PDGF-AA, and cells harvested for RNA isolation at various time points afterwards. The RNA was then subjected to Northern blot analysis, first with a probe to Mhox (see Materials and Methods), and then with a probe to GAPDH, to demonstrate message integrity.
Figure 21: Effect of PDGF-BB on MHOx expression in VSMCs. Quiescent VSMCs were stimulated with 10 ng/ml recombinant PDGF-BB, and cells harvested for RNA isolation at various time points afterwards. The RNA was then subjected to Northern blot analysis, first with a probe to MHOx (see Materials and Methods), and then with a probe to GAPDH, to demonstrate message integrity.
A.

![Image of gel electrophoresis with bands labeled Q, 0.25, 0.5, 1, 2, 4 Hours above and GAPDH to the right]

B.

![Graph showing relative MHist/Perox mRNA level against hours after mitogen]

Figure 22: Effect of PDGF isolated from human platelets on MHist expression in VSMCs. A. Quiescent VSMCs were stimulated with 10 ng/ml PDGF from human platelets, and cells harvested for RNA isolation at various time points afterwards. Such RNA preparations contain all three isoforms of PDGF, with AB predominating. The RNA was then subjected to Northern blot analysis, first with a probe to MHist (see Materials and Methods), and then with a probe to GAPDH, to demonstrate message integrity. B. Quantification of MHist up-regulation. Bands on autoradiograms from Figures 19-22 were quantified by scanning densitometry and normalized to the intensity of the corresponding GAPDH band.
Discussion

Unlike the case of developing skeletal muscle [15, 16], little is known about the transcription factors responsible for the behavior of VSMCs in response to mitogenic stimuli. Although ubiquitous transcription factors such as \( c-fos \) and \( c-myc \) play a role [2, 26, 51], as they do in other cells, it is likely that there exist more tissue-restricted transcription factors which regulate VSMC behavior, including differentiation and dedifferentiation, in response to various growth factors. Knowledge of such factors, besides improving our understanding of the processes behind the development of vascular tissue, could suggest potential targets for gene therapy of disease involving abnormal VSMC proliferation, such as coronary artery restenosis after balloon angioplasty and atherosclerosis [10-12].

Homeobox genes are good candidates for such factors because of their roles in other tissues determining pattern formation and cell fate and regulating tissue-specific gene expression [27-35, 37-40]. One homeobox gene (\( Hoxa-3 \)) is known to be critical in the formation of the heart and aortic arches in mice [28]; another (\( tinman \)) is critical for the formation of cardiac muscle in \( D. \ melanogaster \) [52]; and two other homeobox genes, \( Hoxa-2 \) and \( Gax \), have been shown to be expressed in the cardiovascular system, including vascular smooth muscle [41, 42]. One of these, \( Gax \), is a growth arrest-specific gene [53-56], although its exact function in VSMCs is yet to be determined [41].

Based on these observations, we believed \( M\text{Hox}/\text{Phox} \) to be a good candidate for a transcription factor regulating VSMC growth and development. Further leading us to anticipate that it might play such a role in VSMCs are its reported interaction with the SRF in enhancing its binding to the SRE [44], its ability to bind to an A/T-rich site in the MCK enhancer, a regulatory element that is essential for high levels of
expression of muscle-specific genes, and the restriction of its expression to mesodermal tissues during development [45].

If MHox/Phox plays such a role in VSMCs, it would be likely that its expression might differ during different parts of the cell cycle, as dedifferentiation is an important part of these cells' reentry into the cell cycle [6, 9, 25, 57]. Indeed, this is what we have found. MHox/Phox message levels are strongly, though transiently, upregulated when quiescent VSMCs are stimulated by either serum or PDGF to re-enter the cell cycle. Moreover, this effect also occurs in NIH3T3 fibroblasts, leading to the speculation that this factor might be important in the G0/G1 transition in a variety of mesodermally-derived cells. The time course of this upregulation is rapid, with the maximum effect occurring approximately four hours after mitogen stimulation and fall to near basal within 24 hours, a time course similar to that of the transient upregulation of c-myc in these cells [2], an observation consistent with its interaction with the SRF, which is also upregulated when quiescent cells are stimulated with mitogens [44].

MHox/Phox might also contribute to the switch between the contractile and synthetic phenotype which occurs when VSMCs are stimulated with mitogens [9], perhaps by repressing the expression of genes associated with the differentiated state. Unlike the SRF [58, 59], its upregulation is not super-induced by pretreatment of cells with a protein synthesis inhibitor such as cycloheximide, implying that the mechanism by which it is upregulated is different than the mechanism active in c-fos and the SRF.
Figure 23: *M霍x* is up-regulated by serum growth factors in NIH3T3 cells. Quiescent NIH3T3 cells were stimulated with 10% FCS, and cells harvested for RNA isolation at various time points afterwards. The RNA was then subjected to Northern blot analysis, first with a probe to *M霍x* (see Materials and Methods), and then with a probe to GAPDH, to demonstrate message integrity.
Because of the involvement of VSMCs in the pathogenesis of atherosclerosis, much effort has been expended in studying factors, either released by platelets, macrophages, or endothelial cells, or present in serum, which regulate their proliferation, as well as the receptors which bind them, and the intracellular signaling pathways thus activated. However, the final common pathway of all these factors will be in the nucleus, in the form of transcription factors which respond to these signals, activating and repressing batteries of subordinate genes appropriate to the signal received. Because of its previously described activities, and now because of its regulation by mitogens in VSMCs, it seems likely that Mbox/Phox may be such a factor. It may thus ultimately prove to be a candidate as a target in the gene therapy of atherosclerosis.

References


CHAPTER 6:
A HOMEBOX TRANSCRIPTION FACTOR, GAX, INHIBITS MITOGEN-
INDUCED VASCULAR SMOOTH MUSCLE CELL PROLIFERATION

Introduction

Vascular smooth muscle cells (VSMCs) play an important role in the biology of blood vessels, both in health and in disease [1-7]. In health, they provide structural integrity to the vessel wall; respond to vasoconstrictive and vasodilatory agents to regulate arterial resistance and venous capacitance [5, 8-11]; and secrete extracellular matrix including elastin [12-14]. When blood vessels are damaged, they respond to growth factors released by platelets, endothelial cells, and macrophages, and re-enter the cell cycle to participate in repairing the damage, returning to their normal quiescent state afterward [1, 7, 15]. In disease processes such as atherosclerosis and coronary artery restenosis after balloon angioplasty, regulation of this normal process is disordered, and VSMCs proliferate excessively [1, 4, 5, 7, 15-17], leading to many of the complications of these disease processes, most of which are due to narrowing of the arterial lumen.

Smooth muscle, and especially vascular smooth muscle, is different from other muscle types such as skeletal and cardiac muscle in that it possesses the ability to re-enter the cell cycle in response to appropriate signals. During this process, VSMCs modulate their phenotype [18], essentially reversing their state of differentiation to become more like primordial VSMCs in embryonic blood vessels [1-4, 7, 18-24]. This phenotypic change, which occurs both in vitro when VSMCs are dispersed in primary culture and in vivo when VSMCs proliferate in response to arterial injury, has been
called phenotypic modulation, and the two phenotypes observed in this process are known as the “contractile state” and the “synthetic state” [1, 19]. Contractile state VSMCs are generally quiescent, not migratory, and relatively insensitive to mitogens; express high levels of contractile proteins, including the smooth muscle-specific isoforms of actin and myosin; contract in response to appropriate nervous or hormonal stimuli; and are associated with normal adult blood vessel wall [1]. Synthetic state VSMCs, on the other hand, migrate in response to chemotactic stimuli; proliferate in response to mitogens; do not contract; express low levels of contractile proteins and high levels of nonmuscle isoforms of actin and myosin; and are capable of secreting large amounts of extracellular matrix. They are associated with the repair of arterial injury, developing blood vessels in the embryo, and disease processes in which VSMC proliferation is disordered, such as atherosclerosis [1-4, 6, 7, 13, 14, 18, 21, 24-32].

The manner in which VSMC phenotype changes are coordinated with changes in their proliferative state implies a tight coupling between proliferation and differentiation in these cells. Although much effort has gone into elucidating external signals which influence or control this process and intracellular pathways activated by these signals, little is yet known about what presumably must be the final targets of this process: transcription factors that activate and/or repress the expression of genes necessary for the transition from the quiescent to the proliferative state—or from the “contractile” to “synthetic” state.

Recently, attention has focused on the role of homeobox genes in the cardiovascular system [33-38], and several such genes are now known to be expressed there (see Chapters 2, 3, 4, and 5) [36, 38]. Homeobox genes have long been known to have important roles in regulating axial patterning and organogenesis during development [33-35, 39-48], tissue specific gene expression [40, 47-54], cell migration [55, 56], and cell growth and differentiation [33, 34, 50, 57-60]. These properties
make them especially promising candidates for being transcription factors regulating
VSMC growth and differentiation, especially in light of the observation that the
expression of two such genes, Gax (Chapter 3) [36] and MHOX (Chapter 5), is
regulated by mitogens in VSMCs.

The expression of Gax has been observed to be rapidly down-regulated when
quiescent VSMCs are induced by serum mitogens to proliferate. The time course of this
down-regulation strongly resembles that of the previously described growth arrest-
specific (gas) and growth arrest and DNA damage-inducible (gadd) genes, whose
expression is induced by conditions causing cell growth arrest and is down-regulated by
signals causing cell proliferation, such as stimulation serum growth factors [61-71].
One of these genes, gas1, encodes a membrane protein and has been shown to be a
negative regulator of cell growth when overexpressed in cells by microinjection [65].
Another, gadd 153, appears to be the hamster homologue of the murine gene \textit{CHOP-}
10, a CCAAT/enhancer-binding protein-related gene whose product is a dominant
negative inhibitor of leucine zipper proteins [72] and has been proposed to play a role in
growth arrest induced by DNA damage [63, 67-69]. Lastly, another gadd gene,
gadd45, has been implicated in a cell cycle checkpoint pathway in which its expression
is activated by p53 [73]. Based on these observations and the gas and gadd gene-like
regulation of its expression in VSMCs, we wondered if Gax might be a negative
regulator of cell growth in VSMCs, as gas1 is in fibroblasts. To test this hypothesis,
we expressed Gax as a glutathione S-transferase (GST) fusion protein and introduced it
into quiescent cultured VSMCs by microinjection. Microinjected GST-Gax significantly
inhibited mitogen-induced entry into S-phase by these cells, and this inhibition was
comparable to that caused by microinjection of a GST-MyoD fusion protein. This
inhibition was specific to Gax, dose-dependent, and reversed by the coinjection of a
highly oncogenic ras mutant protein [74]. We conclude that Gax is a negative regulator
of VSMC growth, and thus may represent a potential target for the genetic therapy of disease processes in which disordered VSMC proliferation is a prominent component of the pathogenesis.

**Materials and Methods**

*Production of recombinant proteins*

To produce recombinant proteins for microinjection, the cDNA coding regions for *MyoD* [75], *Gax* [36], and *YY1* [76, 77] were fused in frame to the pGEX-2T expression vector (Pharmacia Biotechnology), expressed in *E. coli*, and the resultant glutathione S-transferase (GST) fusion proteins were purified by affinity chromatography on glutathione-agarose beads.\(^1\) To rule out effects due to contaminating bacterial proteins in the GST fusion protein preparations, the unmodified pGEX-2T expression vector was used to produce the GST protein, and this was also microinjected into cells. Purity of the proteins was >90% as estimated by SDS-PAGE gels stained with Coomassie blue. To produce recombinant *MHox*, its cDNA was fused in frame to the pQE-9 *E. coli* expression vector (Qiagen, Inc., Chatsworth, CA), expressed in bacteria, and purified by adsorption to a nickel column\(^2\) [78]. To verify that the bacterially produced proteins produced were active, gel mobility shift assays were performed using DNA probes containing the binding site for each factor: for *MHox*, the A/T-rich site in the mouse muscle creatine kinase (MCK) enhancer was used as the DNA probe [79]; for *YY1*, the muscle regulatory element (MRE) of the chicken skeletal actin promoter, -100 to -73 nucleotides from the start of transcription; for

\(^1\)Initial production and purification of recombinant GST fusion proteins were performed by Donald Suciu.

\(^2\)Production and purification of histidine-tagged *MHox* protein were performed by Robert Patrick.
MyoD, the right E-box of the mouse MCK enhancer [80]. Because Gax also binds to
the MHox A/T-rich site in the MCK enhancer (albeit with lower affinity) [36], and
because its optimal binding site has not yet been reported, activity of recombinant GST-
Gax was checked by gel shift using this site.

For microinjection, proteins were concentrated in a buffer consisting of 20 mM
Tris, 40 mM KCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, and 2% glycerol using
Centricon-30 (Amicon) microconcentrators. Concentrated proteins were stored in this
buffer in aliquots at -80°C, and the protein concentrations of solutions used in
microinjection experiments were approximately 2 mg/ml. Exceptions included YY1,
whose concentration was 1.2 mg/ml, and Gax, which is relatively insoluble and tended
to form aggregates at concentrations greater than 0.7 mg/ml. Unless stated otherwise,
in most experiments Gax was injected at a concentration of 0.6 to 0.7 mg/ml.

The anti-ras antibody Y13-259 [74, 81, 82] and the oncogenic Ras[Leu-61]
mutant [74] were kind gifts of D. Stacey (Cleveland Clinic Foundation) and were
injected at concentrations of 8 mg/ml and 0.5 mg/ml, respectively, unless otherwise
stated.

Cell culture and reagents

 Cultures of rat thoracic aorta smooth muscle cells were obtained from the media
of aortas isolated from adult male Sprague-Dawley rats by enzymatic digestion
according to previously described methods [83]. Briefly, thoracic aortas were isolated
from Sprague-Dawley rats. The intima and adventitia were carefully stripped, and the
medial smooth muscle layer enzymatically dispersed with trypsin and elastase. Cells
were seeded onto dishes in medium containing a 1:1 mixture of Dulbecco’s modified
Eagle’s medium and Ham’s F12 (DME/F12), supplemented with 10% newborn calf
serum (CS). Once established, the cells were maintained at 37°C in a humidified
atmosphere of 5% CO₂ in DME/F12 with 10% CS, and subcultured within three days after reaching confluence. VSMCs thus cultured grew in a typical hillock and valley pattern and were labeled with monoclonal antibodies to smooth muscle α-actin (Sigma Chemical Co.) to verify identity. Cells were between passages 3 and 8 when used for experiments. BALBc3T3 cells were a kind gift of D. Stacey (Cleveland Clinic Foundation) and were cultured in Dulbecco’s modified Eagle’s medium with 10% CS.

Microinjections and cell proliferation assays

Microinjections were performed using a semiautomatic microinjection system (Eppendorf) in conjunction with a phase contrast microscope (Nikon Diaphot-GMD). Cells were plated onto gridded coverslips and allowed to grow to approximately 50-75% confluence. Prior to microinjection, quiescence was induced by incubating the cells in media containing 0.5% CS for a period of two days (in the case of BALBc3T3 cells) or three to four days (in the case of rat VSMCs). In each experiment, all cells within an area comprised of adjacent squares on the coverslip grid were injected with protein solution. For most experiments, the injection pressure was set between 70-200 hPa and the injection time between 0.3 and 0.6 seconds. Generally, 100 to 200 cells per experimental condition were injected over a period of 15 minutes. In parallel, on the same coverslip, control cells in a nearby area on the grid were injected with microinjection buffer alone. In addition, experimental groups were injected with 2 mg/ml GST protein, or 8 mg/ml goat anti-rabbit IgG (Sigma). For purposes of calculating growth inhibition, microinjection buffer-injected cells (sham-injected) were used as a control. Based on experiments in which fluorescein (FITC)-labeled dextran (Fluorescent Probes, Inc.) was injected into cells, followed by fixation and observation of the cells four to eight hours later, it was estimated that generally 80-90% of cells were successfully microinjected in each experiment.
After injection, cells were stimulated 24 hours with medium containing 10% FCS, and the incorporation of 5'-bromo-2'-deoxyuridine (BrdU) was measured with a cell proliferation kit according to the recommendations of its manufacturer (Amersham). This kit uses a secondary antibody conjugated to horseradish peroxidase to detect nuclei labeled with anti-BrdU antibody, staining them bluish-black. When FCS-stimulated BrdU labeling was determined, BrdU was included for 24 hours with the medium used to stimulate the cells. When the mitotic index of asynchronously cycling cells was determined, BrdU was added to the medium and the cells incubated for four hours. In the case of experiments where the ability of microinjected proteins to stimulate growth in serum-poor medium was measured, cells were incubated 24 hours in the same low serum medium used to induce quiescence, but supplemented with BrdU. After labeling, the cells were fixed with acid-ethanol, and the percentage of nuclei positive for BrdU uptake was determined for protein-injected and buffer-injected cells.

For determination of inhibition of BrdU uptake, the number of injected-labeled cells (IL) was divided by the total number of injected cells (IT). This number was then divided by the ratio of the number of control injected cells labeled (CL) compared to the total number of control injected cells counted (CT). Percent inhibition was thus calculated for each individual experiment according to the following formula:

\[
\% \text{ Inhibition} = \frac{\frac{CL}{CT} - \frac{IL}{IT}}{\frac{CL}{CT}} \times 100
\]

With this equation, inhibition of mitogen-induced entry into S phase is represented by a positive number and stimulation of cell growth is represented by a negative number. For experiments in which the stimulation of growth in quiescent cells by microinjected proteins was determined, the ratio of BrdU labeling in protein-injected
quiescent cells to that in control-injected quiescent cells was used to compare relative efficiencies in inducing cell entry into S-phase.

To identify injected cells, in a subset of experiments proteins were coinjected with FITC-labeled dextran (Fluorescent Probes, Inc.). After incubation in BrdU-containing media, cells were fixed with 4% paraformaldehyde plus 0.2% glutaraldehyde, treated with 1.5 N HCl for 15 min. to denature nuclear DNA, and washed several times with copious volumes of phosphate-buffered saline. Cells were then permeabilized with Triton X-100 and labeled with anti-BrdU antibody from the cell proliferation kit (Amersham), followed by a secondary antibody, Texas Red-conjugated goat anti-mouse IgG2a (Sigma). Cells were then placed under Fluoromount-G (Fisher) and viewed under epifluorescence using appropriate filters for FITC and Texas Red.
TABLE 1: Effect of Microinjected Proteins on Mitotic Index in Asynchronously Cycling VSMCs

<table>
<thead>
<tr>
<th>Protein</th>
<th>Number of Experiments</th>
<th>Total Number of Cells Injected</th>
<th>Mitotic Index ± standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not injected</td>
<td>3</td>
<td>326</td>
<td>0.31 ± 0.05</td>
</tr>
<tr>
<td>Buffer alone</td>
<td>3</td>
<td>208</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>Gax</td>
<td>3</td>
<td>209</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>MyoD</td>
<td>3</td>
<td>147</td>
<td>0.30 ± 0.07</td>
</tr>
<tr>
<td>YY1</td>
<td>2</td>
<td>108</td>
<td>0.24 ± 0.07</td>
</tr>
</tbody>
</table>

Finally, in a subset of experiments, in order to identify when VSMCs enter S phase, cells made quiescent as described above were stimulated with FBS and, at different times after stimulation, were pulsed with $^3$H-thymidine at 5 uCi/ml, and the fold increase in uptake over that observed in quiescent cells determined.

**Results**

*Gax does not inhibit the growth of asynchronously cycling VSMCs*

To test recombinant Gax protein for growth inhibitory properties, it was microinjected into asynchronously cycling cells and the cells were labeled for four hours in BrdU-containing medium (Table 1). A diagram of the apparatus used to perform the microinjections is shown in Figure 24. This semiautomated system permits the injection of 200-300 cells in approximately 15 minutes, enhances reproducibility of injections, and minimizes trauma to the cells due to microinjection.

There was no significant difference between the percentage of VSMC nuclei labeled with BrdU in the sham-injected cells and the percentage in the cells injected with Gax protein (Table 1). This result is similar to that found for microinjected
retinoblastoma protein (Rb), which does not significantly inhibit cell growth in asynchronously cycling Saos-2 cells [84]. Among other proteins injected, neither MyoD nor YY1 had a significant impact on the growth of asynchronously cycling VSMCs as compared to microinjection of buffer alone (Table 1). Similar results were obtained when the same proteins were microinjected into BALBc3T3 cells (data not shown).

We also note that microinjection of buffer alone caused a small but reproducible decrease in the percentage of VSMCs labeling with BrdU both in asynchronously cycling cells, and in serum-starved cells stimulated with serum to enter S-phase synchronously, suggesting that the injections themselves might be harming the cells (Table 1, and notes under Table 2). In early experiments, injecting quiescent cells prior to serum-stimulation with microinjection buffer alone caused decreases as large as 10% in S-phase entry as compared to uninjected cells. In later experiments, this difference was much less, as low as 0-3% in some experiments, but usually approximately 5% (not shown). It should also be noted that this effect was observed whether or not glycerol or β-mercaptoethanol were included in the microinjection buffer (data not shown). We attribute this decrease to increasing familiarity with the technique of microinjection, such that cells in later experiments underwent less trauma during microinjection. BALBc3T3 injected with buffer alone also showed a slight decrease in labeling as compared to uninjected controls (see notes under Table 3), although its magnitude was lower. To compensate for this effect, all calculations of cell growth inhibition or stimulation were performed using the mitotic index of cells from the same coverslip injected with buffer alone (sham-injected), rather than un.injected cells, as the control group.
TABLE 2: Effect of Microinjected Proteins on the Serum-induced Proliferation of VSMCs

<table>
<thead>
<tr>
<th>Protein Injected</th>
<th>Number of Experiments</th>
<th>Total Number of Cells Injected</th>
<th>Mean % Inhibition of FBS-stimulated Growth ± Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody Y13-259</td>
<td>2</td>
<td>328</td>
<td>60.8 ± 3.9</td>
</tr>
<tr>
<td>Mouse anti-human IgG</td>
<td>3</td>
<td>330</td>
<td>-3.4 ± 4.5</td>
</tr>
<tr>
<td>Gax</td>
<td>12</td>
<td>1784</td>
<td>40.6 ± 3.3</td>
</tr>
<tr>
<td>MHOx</td>
<td>2</td>
<td>236</td>
<td>-5.3 ± 9.3</td>
</tr>
<tr>
<td>MyoD</td>
<td>9</td>
<td>1434</td>
<td>39.5 ± 3.7</td>
</tr>
<tr>
<td>YY1</td>
<td>5</td>
<td>306</td>
<td>0.0 ± 12.2</td>
</tr>
<tr>
<td>GST</td>
<td>7</td>
<td>1144</td>
<td>-2.6 ± 2.1</td>
</tr>
</tbody>
</table>

Notes on Table 2: Quiescent VSMCs were microinjected with recombinant protein as described in the Materials and Methods section, then immediately stimulated for 24 hours with 10% FCS in medium containing BrdU. Afterwards, the cells were fixed and labeled for nuclear BrdU incorporation, and the percentage inhibition for each experiment was determined. For these experiments, BrdU labeling of quiescent VSMCs was 10.1 ± 1.2% (N=12, total number of cells counted = 2659); for uninjected FCS-stimulated VSMCs, 54.8 ± 2.4% (N=27, total number of cells counted = 4282); and for sham-injected FCS-stimulated cells, 49.6 ± 2.5% (N=27, total number of cells injected = 3401).

_Gax inhibits mitogen-induced S phase entry in VSMCs and BALBc3T3 cells_

Because _Gax_ has an expression pattern like that of a _gadd_ or _gas_ gene, we wished to determine if it is a negative regulator of VSMC growth. The ability of microinjected Gax protein to inhibit serum-induced VSMC entry into S-phase was therefore tested. Quiescent VSMCs were microinjected with Gax as described in the Materials and Methods section, and then labeled with BrdU in medium containing 10% FCS. After 24 hours, the fraction of nuclei labeling with BrdU was determined and percentage inhibition of S-phase entry calculated for each experiment using sham-injected cells on the same coverslip as controls. For a negative control and to rule out effects due either to the GST part of the fusion proteins injected or to contaminating
bacterial proteins in the GST fusion protein preparations, we also injected GST produced from vector alone. For another control, we injected a mouse IgG against human IgG, which was not expected to influence cell proliferation, a conclusion based on a previous study in which concentrations as high as 10 mg/ml of a similar antibody against IgG had no effect on NIH3T3 cell proliferation [81]. Finally, we injected two other transcription factors. The first was MHOx, a homeodomain protein unlikely to have an inhibitory effect on cell proliferation, a conclusion based on the ability of its human homologue (Phox) to enhance the activity of the serum response factor. This observation suggests that, if anything, MHOx might be expected to stimulate cell growth [85]. The second was YY1 [76, 77], a zinc finger transcription factor that we also considered unlikely to have a negative effect on cell growth.

As a positive control for growth inhibition, a neutralizing antibody against viral ras, Y13-259 [74, 81, 82], was injected. This antibody is highly effective in blocking S phase entry when microinjected into NIH3T3 cells [74, 81], and it was anticipated that it would behave similarly in VSMCs. Because of MyoD’s ability to cause cell growth arrest [75, 86-90], we wished to compare its ability to arrest VSMC growth to that of recombinant Gax, and therefore performed a series of experiments in which recombinant MyoD was injected into VSMCs. The results of these experiments are summarized in Table 2 and Figure 25.
Figure 25: Inhibition of the mitogen-stimulated entry of quiescent VSMCs into S-phase by microinjected Gax and MyoD GST fusion proteins. Quiescent VSMCs were microinjected with recombinant protein as described in the Materials and Methods section, then immediately stimulated for 24 hours with 10% FCS in medium containing BrdU. Afterwards, the cells were fixed and labeled for nuclear BrdU incorporation, and the percentage inhibition for each experiment was determined and averaged as described.

In these experiments, Gax inhibited VSMC entry into S-phase by 40.6 ± 3.3% (Table 2). This represents a slightly greater potency than that of MyoD, whose inhibition of VSMC entry into S-phase in these experiments was 39.5 ± 3.7% (Table 2). In these experiments, MyoD inhibition of mitogen-induced S-phase entry was lower than has been previously reported for the microinjection into fibroblasts of the MyoD cDNA under the control of a constitutive promoter (see Discussion) [88]. However, it is clear from this data that Gax was able to inhibit the mitogen-induced entry of VSMCs
into S-phase at least as well as MyoD. In human VSMCs and fibroblasts, inhibition is slightly more efficient with Gax inhibiting mitogen-induced cell proliferation by 46.6 ± 8.1% and 45.6 ± 0.9%, respectively, in these cell types. Furthermore, this effect on mitogen-stimulated entry into S phase is specific. Other injected proteins failed to inhibit VSMC growth as compared to sham-injected controls (Figure 25 and Table 2), including GST alone, YY1, MHOx, and mouse anti-human IgG.

In comparison to Gax and MyoD, antibody Y13-259, as anticipated, had a dramatic impact on mitogen-induced cell proliferation. VSMCs microinjected with Y13-259 demonstrated a 61 ± 4% decrease in cell entry into S-phase (Table 2 and Figure 25), and BALBc3T3 cells exhibited a 57 ± 21% inhibition (Table 3). This level of inhibition, both in VSMCs and BALBc3T3 cells, is significantly lower than was originally reported for this antibody microinjected into NIH3T3 cells, where it caused a growth inhibition of 78-95% [81]. However, we note that our method of quantifying growth inhibition will tend to underestimate growth inhibition or stimulation, because in most experiments injected cells were not tagged with a coinjected marker and microinjection efficiency was not 100%. We also note that the concentration of antibody Y259 used in our experiments was slightly lower than in the original report, where it was used at a concentration of 10 mg/ml, although 7 mg/ml was sufficient for near-maximal inhibition in NIH3T3 cells [81]. When efficiency of microinjection (80-90% in these experiments) is corrected for, inhibition of VSMC entry into S-phase by microinjection of this antibody approaches 75%, a value closer to that previously reported. Potential reasons for this discrepancy between our results and previously reported results might include differences between cell types in responsiveness to this antibody, differences in microinjection technique and perhaps volume injected per cell, microheterogeneity in both the VSMCs injected (which are not derived from a clonal cell
Figure 26: Dose-response curve for inhibition of mitogen-stimulated VSMC entry into S-phase due to Gax. Quiescent VSMCs were microinjected either with microinjection buffer alone or with Gax, and the percent inhibition of growth determined as described in Materials and Methods. Each point represents the mean ± standard error of 3 to 5 experiments, and with 100-200 cells injected per experiment.

line) and their microenvironment, and possibly differences in antibody half-life in the cytoplasms of different cell types.

Next, we wished to determine what concentration of microinjected Gax is required to inhibit VSMC growth. For this purpose, solutions containing different concentrations of Gax were microinjected into quiescent cells and their effects on mitogen-stimulated entry into S phase examined (Figure 26). The concentration of Gax at which maximal attainable growth inhibition was observed was approximately 0.5 mg/ml, which represents a protein concentration of 7 μM. At the pressure and injection time settings we used, approximately 1-2 pl of solution were usually injected [84], representing approximately 4 to 8 million copies of Gax per injection. Higher
concentrations, up to 1.0 mg/ml, where significant protein aggregation caused micropipet tip clogging, did not significantly improve the level of inhibition observed.

In order to confirm that it was the cells injected with Gax and MyoD whose growth was actually being inhibited, a fluorescent marker (FITC-conjugated dextran) was coinjected with these proteins, allowing injected cells to be identified with certainty. Because coinjection of a marker necessitated diluting the proteins to be injected, and because the dextran/protein solutions tended to clog the micropipet much more readily than protein solutions alone, the majority of experiments were performed without the use of a coinjected marker. However, these experiments confirmed our previous results. Gax inhibited mitogen-stimulated VSMC proliferation by 47 ± 12% (N=3, total number of cells positive for coinjected FITC marker = 238), and MyoD inhibited VSMC growth by 38 ± 2% (N=4, total number of cells positive for coinjected FITC marker = 479), whereas neither YY1, mouse anti-human IgG, nor FITC alone had a significant effect on cell growth, either positive or negative (data not shown).

Finally, we wished to compare the ability of Gax to inhibit growth in VSMCs with its ability to do so in a fibroblast cell line. We therefore injected BALBc3T3 cells with a subset of the proteins with which VSMCs had been injected. These experiments, summarized in Table 3, show that Gax and MyoD also inhibited the mitogen-stimulated entry of quiescent BALBc3T3 cells into S-phase, with comparable efficiencies to those observed in quiescent VSMCs. MyoD appeared to be somewhat more effective at inhibiting the growth of BALBc3T3 cells (growth inhibition = 44 ± 8%, Table 3) than in VSMCs (growth inhibition = 40 ± 4%, Table 2). Antibody Y13-259 also inhibited BALBc3T3 growth as expected, although again it was slightly less potent a growth inhibitor in these experiments as previously reported [81]. Overall, the results obtained with BALBc3T3 cells resemble what was found in VSMCs (Table 2) for the abilities of Gax and MyoD to inhibit cell growth.
TABLE 3: Effect of Microinjected Proteins on the Serum-induced Proliferation of BALBc3T3 Cells

<table>
<thead>
<tr>
<th>Protein Injected</th>
<th>Number of Experiments</th>
<th>Total Number of Cells Injected</th>
<th>Mean % Inhibition of FBS-stimulated Growth ± Standard Error</th>
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</thead>
<tbody>
<tr>
<td>Antibody Y13-259</td>
<td>2</td>
<td>268</td>
<td>56.5 ± 21.3</td>
</tr>
<tr>
<td>Gax</td>
<td>4</td>
<td>464</td>
<td>43.8 ± 10.9</td>
</tr>
<tr>
<td>MHOx</td>
<td>4</td>
<td>400</td>
<td>-3.0 ± 15.1</td>
</tr>
<tr>
<td>MyoD</td>
<td>4</td>
<td>432</td>
<td>43.5 ± 7.6</td>
</tr>
<tr>
<td>GST</td>
<td>2</td>
<td>222</td>
<td>-0.2 ± 1.2</td>
</tr>
</tbody>
</table>

Notes: Quiescent BALBc3T3 cells at approximately 75% confluence were microinjected with recombinant protein as described in the Materials and Methods section, then immediately stimulated for 24 hours with 10% FCS in medium containing BrdU. Afterwards, the cells were fixed and labeled for nuclear BrdU incorporation, and the percentage inhibition for each experiment was determined. For these experiments, BrdU labeling of quiescent BALBc3T3 cells was 5.8 ± 3.1% (N=5, total number of cells injected = 658); for uninjected FCS-stimulated cells, 64.3 ± 3.2% (N =9, total number of cells injected = 1364); and for sham-injected FCS-stimulated cells, 58.1 ± 3.9% (N=9, total number of cells injected = 1150).

_The ras[Leu-61] mutant can reverse growth inhibition caused by Gax_

To define further the growth-inhibitory properties of the Gax protein, we wished to determine if its inhibition of VSMC growth could be reversed by a transforming oncogene. The transforming Ras mutant Ras[Leu-61] is able to cause efficient induction of DNA synthesis in the absence of serum growth factors when microinjected in NIH3T3 cells [74]. In order to test if this mutant could reverse growth inhibition caused by Gax, we performed coinjection experiments, in which a solution containing both Gax and Ras[Leu-61] was microinjected into quiescent VSMCs (Figure 27). In these experiments, when Ras[Leu-61] alone was injected, an increase in mitogen-stimulated BrdU-labeling was observed compared to sham-injected cells, and for cells injected with Gax alone, VSMC growth was inhibited 39% (Figure 27). This value of
Figure 27: Oncogenic Ras[Leu-61] reverses VSMC growth inhibition by Gax in VSMCs. Quiescent VSMCs at approximately 80% confluence were microinjected with either Gax alone (0.5 mg/ml), Ras[Leu-61] alone (0.5 mg/ml), or Gax (0.5 mg/ml) and Ras[Leu-61] (0.5 mg/ml), and then stimulated for 24 hours with 10% FCS supplemented with BrdU as described in Materials and Methods. Cells were also injected with GST (2 mg/ml) as a control. Each data point represents the mean of four independent experiments, in which 100-200 cells per experiment were injected.

Growth inhibition due to microinjected Gax is in agreement with previous experiments (Table 2). When Gax and Ras[Leu-61] were coinjected, Ras[Leu-61] effects predominated, and Ras[Leu-61] completely reversed the growth inhibitory effects of Gax. The percentage of cells staining positive for BrdU in cells coinjected with Ras[Leu-61] and Gax were nearly identical to that observed in VSMCs microinjected with Ras[Leu-61] alone.

Last, we wished to determine if Gax could inhibit growth caused by Ras[Leu-61] in quiescent VSMCs. To this end, the opposite experiment was done; i.e., Gax alone, Ras[Leu-61] alone, or Gax and Ras[Leu-61] together were microinjected into
Figure 28: Ras[Leu-61] induces DNA synthesis in quiescent VSMCs and this effect is not blocked by Gax. Quiescent VSMCs at approximately 80% confluence were microinjected with either Gax alone (0.5 mg/ml), Ras[Leu-61] alone (0.5 mg/ml), or Gax (0.5 mg/ml) and Ras[Leu-61] (0.5 mg/ml), and then incubated for 24 hours in low serum medium supplemented with BrdU as described in Materials and Methods. Cells were also injected with GST (2 mg/ml) as a control. Each data point represents the mean of four experiments.

Quiescent VSMCs, and the percentage BrdU labeling over 24 hours in the same low serum medium used to induce quiescence was determined. As has been observed in NIH3T3 cells [74], microinjected Ras[Leu-61] was capable of inducing a greater than four-fold increase in the rate of DNA synthesis in quiescent VSMCs (Figure 28). The mitotic index of Ras[Leu-61]-injected cells is 40% (data not shown), nearly as high as that observed in serum-stimulated sham-injected VSMCs, which had a mitotic index of 50% (Table 2). Microinjected Gax alone had no significant effect on the background rate of DNA synthesis of VSMCs under the same conditions (Figure 28). Furthermore, when Gax was coinjected with Ras[Leu-61], it failed to inhibit the increase in BrdU labeling induced in quiescent VSMCs by this oncoprotein (Figure 28).
Taken together, these results suggest that, while Gax is able to inhibit VSMC growth caused by normal physiological signals such as serum growth factors, this growth inhibition can be overcome by an oncogenic Ras mutant, which disrupts the normal growth control machinery of the cell and causes uncontrolled cellular proliferation. Thus, Gax activity could represent a normal check on cell growth, probably one of many. Equally important, these experiments (Figure 27 and 28) tend to argue against toxicity as a cause of growth inhibition by Gax. If the Gax growth inhibition observed in early experiments (Table 2 and Figure 25) were due to its toxicity relative to other proteins microinjected, then it would be likely to inhibit growth caused by the oncogenic Ras[Leu-61] mutant, because inhibition of growth would be due to cellular dysfunction. Gax did not significantly decrease the ability of Ras[Leu-61] to induce DNA synthesis in quiescent cells when microinjected at concentrations which normally cause maximal inhibition of serum-induced DNA synthesis (Figure 28). Also, Ras[Leu-61] was able to reverse completely the inhibition of serum-induced DNA synthesis by Gax, such that cells coinjected with Gax and Ras[Leu-61] exhibited growth indistinguishable from cells injected with Ras[Leu-61] alone. These two results are not consistent with a mechanism of growth inhibition by Gax due to cellular toxicity.

**Gax inhibits cell growth when microinjected before the G1/S boundary**

In order to attempt to determine where in the cell cycle Gax might exert its growth inhibitory effects, the time of S phase onset was determined in VSMCs, and then these cells were microinjected with GST-Gax at various times after stimulation with FBS and labeled with BrdU between 10 and 24 hours after stimulation. Percent inhibition of S-phase entry was determined at each time point. Gax caused significant inhibition of VSMC entry into S phase when microinjected at any time until approximately 12 hours, whereas the cells were clearly entering S-phase by
approximately 15 hours (Figure 29), a time course in agreement with what has been previously reported for this cell type [91]. These observations imply that Gax likely inhibits a critical step in cell cycle progression late in G1 or at the G1/S boundary.

![Graph](image)

Figure 29: Gax inhibition of VSMC proliferation when microinjected at different times in the cell cycle. The time of the onset of S-phase in rat VSMCs was determined by pulsing cells with 3H-thymidine at different times after mitogen stimulation. To determine when in the cell cycle microinjected Gax ceases to be effective in inhibiting VSMC growth, quiescent VSMCs were microinjected with GST-Gax (0.6 mg/ml) and then stimulated with FBS. Subsequently, other cells on the same coverslip were injected at different times after FBS stimulation and the cells on the coverslip labeled with BrdU between 10 and 24 hours after stimulation. Key: Each data point represents the mean of three measurements. Key: Squares (left-hand Y axis) represent percent inhibition of S-phase calculated as described in Materials and Methods; circles (right-hand Y axis) represent fold-increase in 3H-thymidine uptake over that observed at time zero. For measurements of percent inhibition, each data point represents the mean of three measurements; for 3H-thymidine uptake, two to five measurements.
Discussion

Growth control in mammalian cells involves a fine balance between the activities of growth promoting genes and growth suppressing genes. Until recently, most attention has been focused on genes which promote growth, and which can be oncogenic, either when they are overexpressed in cells or when mutations alter normal checks on their function. However, in the last few years the importance of genes that inhibit cell growth has become more and more recognized. Several genes have been described whose expression is induced by signals causing cell growth arrest, such as the growth arrest-specific (gas) genes [64-66, 70, 71] and the growth arrest and DNA-damage inducible (gadd) genes [61, 63, 67-69]. These genes are thought to be involved in growth suppression, and for at least one of them, gas1, direct experimental evidence exists for its role in arresting cell growth [65]. One of the gadd genes, gadd153, is known to encode a CCAAT/enhancer binding protein (C/EBP)-related protein whose expression is induced by DNA-damaging agents and other signals which normally cause cell growth arrest [61, 63, 67-69]. While unable to bind DNA, gadd153 can probably dimerize with other C/EBP proteins through its leucine zipper motif and inactivate them. Such a mechanism of action has been demonstrated for a gene that is probably its murine homologue, CHOP-10 [72]. The expression of another gadd gene, gadd45, depends upon normal p53 expression and its induction in response to growth arrest and DNA damage is defective in ataxia-telangiectasia [73]. It is not unreasonable to speculate that a growth arrest-specific transcription factor like Gax, whose expression is regulated in a manner very similar to that of the gas and gadd genes [36], might influence the growth state of the cell, likely through a mechanism in which it either activates the expression of genes appropriate to the quiescent state or represses the expression of genes appropriate to the proliferating state.
Positive and negative regulators of VSMC growth are of particular interest because of the relevance of VSMC growth control to human disease states whose pathogenesis involves uncontrolled or disordered growth of vascular myocytes, such as atherosclerosis, coronary artery restenosis after balloon angioplasty, and the failure of vascular grafts [7, 15, 17, 29, 30, 92]. VSMCs modulate their phenotype when they re-enter the cell cycle, changing their state of differentiation to resemble that of vascular myocytes in developing blood vessels in the embryo [1, 7, 17, 19, 20, 29, 30, 93]. In the study of this aspect of vascular myocyte biology, external signals which influence the growth of this cell type, such as growth factors, cytokines, and extracellular matrix attachments, have been extensively studied [1-3, 7, 10, 17, 19, 20, 22, 27-30, 94-96]. Presumably, the ultimate targets of these signals are transcription factors whose activity they increase or decrease and which activate or repress the expression of target genes appropriate to the signal. It is then the increases or decreases in the expression and/or the activities of these target genes that determine the growth state of the cell. Because homeobox genes represent a class of transcription factors known to be involved in pattern formation, tissue-specific gene expression, and organogenesis [34, 35, 39-41, 43-46, 48-54, 57-59, 85, 97-101] and are expressed in a wide variety of evolutionarily distant organisms [42, 45, 48, 99, 102-109], they would appear to be excellent candidates for a role in coordinating growth and differentiation in VSMCs, both in normal and disease states.

We first suspected Gax to be a negative regulator of growth on the basis of its expression pattern in VSMCs [36], which strongly resembled that of previously described gas and gadd genes [62, 64-66, 70, 71]. Like that of gas and gadd genes [64, 66, 71], Gax expression is highest in quiescent cells and is rapidly down-regulated when VSMCs re-enter the cell cycle in response to serum mitogens. Its expression is also induced when sparsely plated proliferating cells undergo growth arrest due to
serum deprivation [36]. Based on these observations and the examples of gas1 and gadd 153, we wondered if Gax might be able to inhibit the growth of vascular myocytes. This determination would be especially interesting because Gax encodes a transcription factor that possesses both an activation and a repression domain (K. Guo and K. Walsh, unpublished result) and binds to a consensus site containing an A/T-rich region at its core (C. Patel and K. Walsh, unpublished result). Identification of downstream targets and Gax's effect on their expression could shed light on the mechanisms by which VSMC differentiation and proliferation are coordinated.

Microinjection of somatic cells is a technique that allows the direct introduction of purified macromolecule solutions into individual cells [110-114]. It is possible to inject DNA, RNA, or protein into cells and observe the effect on cell function, as long as an assay exists that can determine information at the level of the individual cell. Most commonly, the properties measured include cell proliferation (as represented nuclear labeling with 3H-thymidine or BrdU), cell morphology, or alterations in the expression of proteins for which antibodies exist. Consequently, microinjection has been used for demonstrating the effect of several oncogenes and tumor suppressors on cell proliferation, including ras mutants [74, 81, 82], Rb [84], MyoD [88], gas1 [65], and E2F [115], to name a few examples. Using this technique, in this study we have obtained evidence that Gax does indeed inhibit VSMC growth. Moreover, it does so specifically, and its effects are completely reversed by coinjection of a highly oncogenic protein, a result that tends to argue against a toxic mechanism of action.

It is not surprising that Gax should inhibit the growth of VSMCs, given the manner in which its expression is regulated by mitogens and growth-arrest signals in VSMCs [36]. Moreover, by this assay it is at least as potent as MyoD in VSMCs at inhibiting growth, although neither protein was able to inhibit VSMC proliferation by
more than 50% in any experiment (data not shown). In comparison, a neutralizing antibody directed at c-ras was able to inhibit cell growth by more than 60% (Table 2). It is possible that technical reasons, such as underestimating the percentage of injected cells, is responsible for not achieving higher levels of inhibition of the Go/S transition in VSMCs, as compared to the previously reported inhibition of mitogen-stimulated proliferation of 86% [81]. Based on experiments in which a fluorescent marker was injected into cells, it was found that between 80-90% of the cells were successfully injected. The least favorable figure of an 80% injection efficiency would imply that the true level of inhibition is approximately 25% higher that what was observed in these experiments, or approximately 75% in the case of experiments with anti-ras antibodies. However, control experiments using a coinjected fluorescent marker to tag injected cells demonstrated a level of inhibition by Gax only slightly higher than when such a marker was not used, an indication that the efficiency of microinjection was high, at least in this subset of experiments. On the other hand, microinjection of antibody Y13-259 into BALBc3T3 cells produced only 57% inhibition, implying that the difference might be due to differences in technique, and that perhaps the true efficiency of microinjection is lower than suggested by experiments with injected fluorescent markers, perhaps due to protein clogging or the injection of less volume (and hence less protein).

In considering the results obtained by this particular assay for Gax and MyoD, the activities of the injected proteins and heterogeneity of the VSMC population microinjected have to be considered. It is also possible that Gax requires post-translational modifications for full activity, modifications that bacterially produced proteins do not undergo. Indeed Gax has multiple consensus sites for phosphorylation by protein kinases [36], and it is possible that its activity is activated or otherwise modulated by phosphorylation at one or more of those sites. The GST fusion might
also have affected the activities of the injected proteins. Although the GST fusion proteins were able to bind DNA with comparable affinities to those of the wild-type (not shown), it is unknown what effect GST might have on other protein functions, such as transcriptional activation or repression. Another potential factor in Gax effectiveness in inhibiting S phase entry in VSMCs is the heterogeneity of these cells. VSMCs in different vascular beds have different embryological origins, leading to microheterogeneity among them in how they respond to mitogens and various other factors [18], and this might explain in part why 40% inhibition was observed, both with microinjected Gax and MyoD proteins. The cells injected do not represent a clonal population, and that, even with stimulation by FBS, only 55% enter S phase, meaning that 45% did not respond to powerful mitogenic signals, and these cells become asynchronous only one cycle after mitogen stimulation, implying very different durations of the cell cycles of individual cells [91]. It is thus possible that only a subset of these cells are capable of responding to the microinjected Gax protein and that this is reflected in the 40% inhibition of cell proliferation observed. Also, as was mentioned before, when corrected for the least favorable injection efficiency, inhibition of mitogen-stimulated VSMC proliferation by Gax approaches 50%.

In assessing the effectiveness of Gax in inhibiting VSMC growth, one can compare its efficacy with that reported for other negative regulators of cell growth. In experiments where antibodies to cell proteins involved in cell cycle progression have been injected, higher efficiencies of inhibition than in the present study have usually, but not always, been observed. For instance, as mentioned previously, microinjected anti-ras antibody inhibited NIH3T3 cell proliferation by 78-95% [74, 81]. However, in another experiment, it was found that antibodies to c-jun and c-fos could effectively inhibit mitogen-induced cell growth in Swiss 3T3 cells (90% inhibition) when polyclonal antibodies that recognized all members of the c-jun and c-fos families were
injected. However, specific antibodies to individual members of the c-fos or c-jun families were much less effective (30-40%), suggesting that either each member could substitute for the others, at least in part, in cell cycle progression or that all cooperated in inducing S-phase entry [116]. It is unlikely that Gax is the only regulator of VSMC growth, and it is possible that it cooperates with other factors to induce growth inhibition. Finally, microinjection of negative regulators of cell growth have also reported efficient inhibition. In Saos-2 cells, microinjected Rb protein produced greater than 90% inhibition of growth when injected into quiescent cells prior to serum stimulation [84], and in NIH3T3 cells a dominant negative ras mutant produced a 71% inhibition [74]. In other experiments, microinjection of a plasmid overexpressing gas1, a growth arrest-specific gene, produced 48-64% inhibition in NIH3T3 fibroblasts [65], and a plasmid overexpressing MyoD1 inhibited cell growth by 73% (see below) [88]. Clearly, the inhibition of VSMC growth produced by microinjected Gax protein is less than observed with Rb or dominant negative ras mutants, but is nearly as high as that observed with another growth arrest-specific gene, gas1.

Another possible explanation for the observation of only 40-50% growth inhibition could be lability of microinjected Gax. Microinjected proteins have half-lives in the cell ranging from hours to days [117]. For instance, microinjected antibodies can remain uncleaved in cells for periods of days [117], and we could detect a mouse IgG directed against human IgG in VSMCs at least 24 hours after injection (data not shown), while microinjected bovine serum albumin has been observed to enter lysosomal granules within three hours after injection [117]. The half-life of Gax within VSMCs is unknown. However, based on the rapid down-regulation of its transcript, we would speculate that its half-life is likely to be relatively short, perhaps on the order of a few hours, if the mitogen-induced down-regulation of its transcript were expected to translate into a down-regulation of the Gax protein. Thus, it is possible that a vector
overexpressing Gax could produce greater inhibition, as Gax protein levels could achieve a steady state within the cell, rather than decaying exponentially according to the protein's half-life. We note that when a plasmid containing the MyoD1 cDNA under the control of an inducible promoter is injected into NIH3T3 cells, growth inhibition as high as 73% was observed in cells shown to be expressing MyoD protein [88].

Analogous experiments for Gax would require a reliable antibody in order to identify injected cells overexpressing Gax protein. Further confounding the matter is the endogenous expression of Gax in VSMCs [36]. It is sometimes possible to overcome such difficulties by using a co-injected vector expressing β-galactosidase to label injected cells that are successfully expressing protein from microinjected plasmid. In such an approach, the investigator must assume that the protein of interest is also being expressed, along with β-galactosidase. Indeed, this is the approach recently used to demonstrate the ability of E2F to cause quiescent NIH3T3 cells to enter S phase [115]. Unfortunately, we were unable to observe β-galactosidase activity in a sufficient percentage of VSMCs co-injected with a β-galactosidase expression vector and the Gax cDNA in the pCB6+ expression vector to make definite conclusions regarding Gax's ability to inhibit VSMC proliferation (data not shown).³

Finally, it is possible that this result may represent the true activity of Gax in inhibiting VSMC growth (i.e., 50% inhibition, correcting for efficiency of microinjection). Coordination of VSMC growth and differentiation undoubtedly depends upon many genes, some growth inhibitory like Gax and others growth-stimulatory. It may be that Gax requires other factors for activity, but that at sufficiently high concentrations it is able to inhibit VSMC growth. Alternatively, it is possible that, if Gax requires other factors, microinjected Gax is sufficient to saturate

³Experiments performed in collaboration with D. Stacey (Cleveland Clinic Foundation).
the system and further Gax activity is not possible without the increased expression of more of these factors, whose expression might presumably be repressed by growth signals. Finally, even with the automated microinjection system, the volume of protein solution injected per cell varies by three-fold or even more [111]. It is thus possible that an unknown percentage of cells were not injected with a sufficient mass of Gax to cause growth inhibition, and, due to its relative insolubility, we were not able to concentrate Gax sufficiently to overcome this limitation. However, we note that growth inhibition in VSMCs due to Gax was comparable, even slightly more marked, than that caused by MyoD, a protein known to be capable of inducing growth arrest and terminal differentiation along the myogenic pathway when overexpressed in nonmuscle cells [80, 86-90]. This suggests that Gax might have a more potent growth inhibitory activity in VSMCs in vivo. In any case, its ability to inhibit VSMC growth makes Gax an attractive target for the genetic therapy of blood vessel diseases whose pathogenesis involves the excessive proliferation of these cells, such as atherosclerosis. It is also likely that identification of its downstream targets will lead to a greater insight into the mechanisms by which VSMC growth and differentiation are coordinated.

References


75. Lassar, A. B., B. M. Paterson, and H. Weintraub. 1986. Transfection of a DNA locus that mediates the conversion of 10T1/2 cells into myotubes. *Cell* 47: 649-656.


CHAPTER 7:

DISCUSSION/FUTURE DIRECTIONS

Control of the proliferative state in mammalian cells is a complex process involving the interplay of growth factors, extracellular matrix components, paracrine and autocrine regulation, intracellular signaling pathways, and transcription factors responsible for activating and repressing the expression of subordinate batteries of genes appropriate for the proliferative or quiescent state. VSMCs are undoubtedly no different. Unlike other types of muscle, vascular smooth muscle cells (VSMCs) retain the ability to re-enter the cell cycle in response to mitogenic stimuli, and it is this property that allows them to participate in the repair of injury under normal conditions [1-7]. These cells also perform other functions, including maintaining vascular tone and regulating systemic vascular resistance, the main determinants of systemic blood pressure, and secreting extracellular matrix [6-10]. When VSMCs re-enter the cell cycle, they modulate their state of differentiation, becoming more like the primitive VSMCs found in embryonic blood vessels during development [1, 2, 4, 5] in that they down-regulate the expression of contractile proteins, especially the smooth muscle-specific forms of actin and myosin, and re-express fetal isoforms of these genes [2-5, 11-13].

How VSMCs coordinate their differentiation and growth has been the topic of intense research for many years because of the potential insights this research can yield into human diseases in which VSMC growth and differentiation are disordered. Such diseases, in the form of atherosclerotic coronary artery disease and cerebrovascular
disease, are the leading cause of mortality in developed countries, accounting for nearly half of all deaths [14]. They are also the cause of significant morbidity, primarily in the form of myocardial infarctions, gangrene due to peripheral vascular disease, and strokes. Twenty years ago, Ross proposed that the mechanism of pathogenesis in atherosclerosis is chronic injury to the endothelium, which causes the release of cytokines and growth factors from the injured endothelial cells, leading to a chronic inflammatory reaction [15]. These factors lead to monocyte adhesion and release of monocyte factors and also stimulate medial VSMCs to migrate to the intima and to proliferate [6, 7, 16, 17]. Sources of injury implicated in this process include hyperlipidemia and abnormal lipoprotein metabolism [7, 18-21], turbulent blood flow [22-24], smoking [25], and hypertension [7, 24]. Although other cell types, including endothelial cells and monocytes, are undoubtedly very important in inciting and maintaining inflammatory reaction at the heart of these disease processes, it is the vascular myocyte whose abnormal proliferation contributes most to the complications of these diseases, most of which are due to compromised blood flow through an affected artery due to the impingement of atherosclerotic plaques [6, 7]. Consequently, the regulation of VSMC proliferation is of intense interest and critical to understanding and designing treatments for these blood vessel diseases.

Research on VSMC growth has focused on external factors that regulate VSMC growth and differentiation. Significant progress has been made in understanding these signals, VSMC responses to them, and intracellular signaling pathways within VSMCs. Much less is known about the molecular biology behind this process. Because of our interest in skeletal muscle development, as well as our interest in transcription factors, we decided to attempt to determine what transcription factors might be important in VSMC growth and development. As discussed in Chapter 1, VSMCs exist somewhere in a spectrum between two phenotypes, the synthetic
phenotype and the contractile phenotype. The contractile phenotype is associated with
normal quiescent VSMCs in the adult organism, and the synthetic phenotype is
associated with proliferating VSMCs in the repair of arterial injury, blood vessel
development in the embryo, and diseases in which VSMC proliferation is disordered,
such as atherosclerosis. VSMC proliferation in response to injury due to balloon
angioplasty, and vascular graft failure [1, 2, 4, 5]. It appears that VSMCs must
convert to the synthetic phenotype in order to become competent to re-enter the cell
cycle [1, 2, 26]. We reasoned that the end result of external signals that change the
state of growth and differentiation in VSMCs between the proliferative and synthetic
states must be alterations in the expression of genes necessary for each state. Further,
such a factor, if it could be demonstrated to influence or regulate VSMC growth, might
prove to be a target for genetic therapy of these disease states perhaps in the way that c-
myb antisense can inhibit VSMC proliferation in response to balloon catheter injury in
vivo [27].

In deciding which classes of transcription factors are most likely to be involved
in phenotypic modulation and regulation of vascular myocyte growth and
differentiation, it was natural to seek an analogy with a related tissue type, skeletal
muscle, where master regulatory genes of the helix-loop-helix (HLH) family of
transcription factors induce cell growth arrest and terminal differentiation [28-33].
However, attempts to isolate these factors from vascular cDNA libraries in this
laboratory have been largely unsuccessful. Although failure to isolate such factors
from vascular tissue does not rule out their expression—or importance—there, we
noted that HLH proteins, at least in muscle, are responsible for inducing terminal
differentiation. Vascular myocytes, on the other hand, remain much more plastic and
are able to modulate their state of differentiation and growth according to external
signals. Thus, it seems likely that HLH proteins are not as critical to vascular smooth muscle growth and differentiation as they are in skeletal muscle [28-35].

Homeobox genes represent a class of transcription factors known to be important in pattern formation in the embryo, organogenesis, expression of cell type-specific genes, and control of cell proliferation [36-52]. With the exception of Hox-1.5, null mutations of which cause severe cardiac and vascular anomalies [37], there was very little known about the potential involvement of homeobox genes in cardiovascular growth, development, and disease states. Consequently, we decided to look for the presence of these factors in vascular tissue, and were successful in isolating several of them. The principal conclusions of this study include the following:

1. Several homeobox genes belonging to the HOX-1 and HOX-2 clusters are normally expressed in vascular myocytes (Chapters 2 and 3) [53, 54].

2. One homeobox gene expressed in VSMCs, Gax, is a diverged homeobox gene related to Max-1. The regulation of its expression is similar to that of the growth arrest-specific (gas) and growth-arrest and DNA damage-inducible genes (gadd) genes, genes whose expression is induced by growth arrest signals and rapidly down-regulated when quiescent cells are stimulated with mitogens [53].

3. No other homeobox gene known thus far to be expressed in VSMCs is regulated in this manner, although the expression of MHox [55, 56] is regulated in the opposite manner; i.e., it is rapidly up-regulated when quiescent VSMCs are stimulated with serum (Chapter 5).
4. As predicted by the manner in which its expression is regulated, Gax is a negative regulator of growth in VSMCs as demonstrated by the observation that microinjected Gax protein inhibits the entry of VSMCs into S phase due to mitogenic stimulation (Chapter 6). As such, it may have the potential to serve as an effective target for the genetic therapy of diseases in which VSMC proliferation is abnormal, such as atherosclerosis, coronary artery restenosis, and the failure of vascular grafts.

The most pressing question involves elucidating the mechanism of VSMC growth inhibition by Gax. Information most likely to shed light on this mechanism would be the identification of Gax's downstream targets and how Gax regulates their expression. Ongoing work in our laboratory indicates that the Gax protein binds to a DNA site with an A/T-rich core (C. Patel and K. Walsh, unpublished result). Given the manner in which VSMCs coordinate their state of differentiation with their proliferative state [2, 3], it would not be surprising if Gax were shown to activate the expression of tissue-specific genes, such as that for the smooth muscle α-actin. Downstream targets whose expression we speculate, Gax might repress include genes associated with entry into S phase, such as that for ornithine decarboxylase or thymidine kinase, both of which are not expressed in quiescent VSMCs but are up-regulated when these cells re-enter the cell cycle [57]. Once the Gax protein consensus binding sequence is determined, promoters containing this sequence can be tested for trans-activation by Gax in tissue culture.

Another approach to determining how Gax is involved in coordinating proliferation and differentiation is to determine what factors are responsible for regulating its expression in VSMCs. For this purpose, the isolation of the Gax promoter is necessary. Indeed, this is another ongoing project in our laboratory, and
we have isolated—and are presently sequencing—a genomic clone that contains the 5’ end of Gax and considerable upstream sequence. Once this promoter is isolated, it can be mapped and the elements important for activating Gax expression identified. Identification of these elements could then, in turn, lead to the identification of transcription factors responsible for regulating Gax expression and help clarify whether the mechanism of Gax down-regulation is transcriptional or post-transcriptional, a point not settled by studies in which the effect of transcriptional inhibitors on this down-regulation was studied (Chapter 3).

It is also of interest to determine Gax’s functional role during embryogenesis.

To this end, the production of mice transgenic for a null mutation in the Gax gene would be the most appropriate approach. The phenotype of mice homozygous for this effect could then be determined. Tissues whose normal development might require Gax expression include the cardiovascular system, the limb buds, the lens, the central nervous system, and possibly even skeletal muscle, as these are all tissues in which Gax is strongly expressed at different times during embryonic and fetal development.

Finally, the other homeobox genes expressed in VSMCs should not be forgotten. One, MHox [55, 56], is regulated in exactly the opposite manner in VSMCs and might potentially be a positive regulator of VSMC proliferation. Other members of the HOX clusters expressed in VSMCs include Hox-1.3, Hox-1.4, Hox-1.11, and Hox-2.3. None of these genes were regulated by mitogens in the way that Gax and MHox were (Chapter 3), but that does not rule out an important role for them during cardiovascular development or in the differentiation of VSMCs. Also, it is highly unlikely that the homeobox genes isolated in this study represent the only homeobox genes expressed in vascular tissue. Of note, Hox-1.5, the only homeobox gene for which functional evidence exists for a role in cardiovascular development [37], was not
isolated from the adult rat VSMC cDNA library. Although Hox-1.5-like homeobox genes represent a distinct class from Antennapedia-like genes [49], they are identical at the amino acid level in the third helix, and thus the H3I probe, which was designed to recognize the third helix (Chapter 2), should have been able to recognize the Hox-1.5 cDNA, were it present.

Prior to this study, almost nothing was known about the potential role of homeobox genes in the cardiovascular system, although their roles in regulating tissue-specific gene expression, cell growth, and pattern formation had been more thoroughly studied in other tissues [36-40, 43, 47-49, 51, 58-62]. Concurrently with this study, evidence of the importance of these factors in the development of the cardiovascular system was reported for Hox-1.5 in the mammalian cardiovascular system [37] and for tinman in Drosophila [63, 64]. Now, we present evidence that several of these genes are expressed in cardiovascular tissues in the adult organism and that one of them, Gax, has a role in the control of the proliferative state of VSMCs (Chapter 6). Because of its inhibitory effect on VSMC growth, Gax is a tempting target for future genetic therapy of diseases involving the abnormal proliferation of VSMCs. It is anticipated that future studies will demonstrate that Gax and other homeobox genes are critical factors regulating development, growth, and differentiation in the cardiovascular system.

References


APPENDIX I: HOMEOBOX GENE NOMENCLATURE

Because of the increasing number of homeobox genes being reported in the literature, a change in vertebrate homeobox nomenclature has been proposed by the Beechwood Ridge Nomenclature Committee in order to systematize both the naming of new homeobox genes and how the old names relate to a gene’s location in the major HOX clusters [1]. Since this system is relatively new and the older names for homeobox genes still predominate in the literature, I decided to use primarily the old nomenclature, with the new names in parentheses when they first appear, in order to avoid confusion among those who might be more familiar with the older nomenclature. However, for completeness, I present in this Appendix a listing of the new names of genes belonging to the four major HOX clusters and the old names that they will replace, a list adapted from Scott [1].
## TABLE 4: New Hox Gene Names

<table>
<thead>
<tr>
<th>Gene</th>
<th>Synonyms</th>
<th>Gene</th>
<th>Synonyms</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1F, 1.6, era-1, l-y, XHoxlab-2</td>
<td>C4</td>
<td>3E, 3.5, cp19, R3</td>
</tr>
<tr>
<td>A2</td>
<td>1K, 1.11</td>
<td>C5</td>
<td>3D, 3.4, cp11, 6.2, XlHbox-5</td>
</tr>
<tr>
<td>A3</td>
<td>1E, 1.5, mo10</td>
<td>C6</td>
<td>3C, 3.3, c8, 6.1, XlHbox-1</td>
</tr>
<tr>
<td>A4</td>
<td>1D, 1.4, HBT-1, MH-3, R2</td>
<td>C8</td>
<td>3A, 3.1, moea, m31, R4</td>
</tr>
<tr>
<td>A5</td>
<td>1C, 1.3, m2</td>
<td>C9</td>
<td>3B, 3.2</td>
</tr>
<tr>
<td>A6</td>
<td>1B, 1.2, m5</td>
<td>C10</td>
<td>3I, 3.6</td>
</tr>
<tr>
<td>A7</td>
<td>1A, 1.1, m6, Xhox-36, R5</td>
<td>C11</td>
<td>3H, 3.7</td>
</tr>
<tr>
<td>A9</td>
<td>1g, 1.7, Chox-1.7</td>
<td>C12</td>
<td>3F, 3.8</td>
</tr>
<tr>
<td>A10</td>
<td>1H, 1.8, PL</td>
<td>C13</td>
<td>3G, 3.9</td>
</tr>
<tr>
<td>A11</td>
<td>1I, 1.9, Chox-11</td>
<td>D1</td>
<td>4G, 4.9</td>
</tr>
<tr>
<td>A13</td>
<td>1J, 1.10</td>
<td>D3</td>
<td>4A, 4.1, R6</td>
</tr>
<tr>
<td>B1</td>
<td>21, 2.9, GHox-lab, X2.9</td>
<td>D4</td>
<td>4B, 4.2, c13, 5.1, Chox-a</td>
</tr>
<tr>
<td>B2</td>
<td>2H, 2.8, K8</td>
<td>D8</td>
<td>4E, 4.3, 5.4, Chox-m</td>
</tr>
<tr>
<td>B3</td>
<td>2G, 2.7, X2.7, Chox-2.7</td>
<td>D9</td>
<td>4C, 4.4, 5.2, Chox-4.4</td>
</tr>
<tr>
<td>B4</td>
<td>2F, 2.6, Chox-Z, Xhox-1a</td>
<td>D10</td>
<td>4D, 4.5, 5.3, Chox-4.5, Chox-4d</td>
</tr>
<tr>
<td>B5</td>
<td>2A, 2.1, H24.1, Mu-1, Hu-1</td>
<td>D11</td>
<td>4F, 4.6, 5.5, Chox-4.6, Chox-4e</td>
</tr>
<tr>
<td>B6</td>
<td>2B, 2.2, mh-22a</td>
<td>D12</td>
<td>4H, 4.7, 5.6, Chox-4.7, Chox-4f</td>
</tr>
<tr>
<td>B7</td>
<td>2C, 2.3, cl, R1b</td>
<td>D13</td>
<td>4I, 4.8, 5.7, Chox-4.8, Chox-4g</td>
</tr>
<tr>
<td>B8</td>
<td>2D, 2.4, XlHbox-7, R1a</td>
<td>Msx1</td>
<td>Hox7, Hox-7.1</td>
</tr>
<tr>
<td>B9</td>
<td>2E, 2.5, XlHbox-6</td>
<td>Msx2</td>
<td>Hox8</td>
</tr>
<tr>
<td>Evx1</td>
<td>Xhox-3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: The first two synonyms refer to previous human and mouse names, respectively. Genes preceded by an X are from *Xenopus laevis*, R from rat, and C from chicken.

### References

APPENDIX II: ADDITIONAL MICROINJECTION EXPERIMENTS

Effect of Microinjected Proteins on NIH3T3 Cell Proliferation

Experiments were also carried out in which recombinant proteins were microinjected into NIH3T3 cells. However, because these cells had a high mitotic index, even after two days of serum deprivation (0.26 ± 0.02), it is difficult to assess how reliable these results are, given the very high background for mitogenesis in these cells. The data are presented here for completeness in Table 5.

TABLE 5: Effect of Microinjected Proteins on NIH3T3 Cell Proliferation

<table>
<thead>
<tr>
<th>Protein Injected</th>
<th>Number of Experiments</th>
<th>Total Number of Cells Injected</th>
<th>Mean % Inhibition of FBS-stimulated Growth ± Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gax</td>
<td>4</td>
<td>420</td>
<td>23.2 ± 1.8</td>
</tr>
<tr>
<td>MyoD</td>
<td>3</td>
<td>273</td>
<td>30.8 ± 3.7</td>
</tr>
<tr>
<td>Rb</td>
<td>2</td>
<td>352</td>
<td>16.2 ± 14.2</td>
</tr>
<tr>
<td>Rb-C706F</td>
<td>2</td>
<td>224</td>
<td>-8.5 ± 0.1</td>
</tr>
<tr>
<td>YY1</td>
<td>1</td>
<td>96</td>
<td>19.6</td>
</tr>
</tbody>
</table>

Notes on Table 2: Quiescent NIH3T3 cells were microinjected with recombinant protein as described in the Materials and Methods section, then immediately stimulated for 24 hours with 10% FBS in medium containing BrdU. Afterwards, the cells were fixed and labeled for nuclear BrdU incorporation, and the percentage inhibition for each experiment was determined. For these experiments, BrdU labeling of quiescent NIH3T3 cells was 26.2 ± 1.6% (N = 2, total number of cells counted = 381); for uninjected FBS-stimulated NIH-3T3 cells, 70.4 ± 2.1% (N = 12, total number of cells counted = 1169); and for sham-injected FBS-stimulated cells, 62.9 ± 2.1% (N = 10, total number of cells injected = 1104).
Effect of Microinjected Rb and Rb-C706F on VSMC and BALBc3T3 Cell Proliferation

A series of experiments was also performed in which recombinant GST fusion proteins of Rb and Rb-C706F proteins were microinjected into VSMCs and BALBc3T3 cells. In these experiments, Rb appeared to demonstrate a slight inhibition of growth, but it was very low and overall, the results of these experiments were inconclusive, especially in light of the fact that we have no assay to determine if our GST-Rb fusion proteins were active, unlike the case with our transcription factors. The results of these experiments are summarized in Tables 6 and 7.
TABLE 6: Effects of Microinjected Rb Proteins on VSMC Proliferation

<table>
<thead>
<tr>
<th>Protein Injected</th>
<th>Number of Experiments</th>
<th>Total Number of Cells Injected</th>
<th>Mean % Inhibition of FBS-stimulated Growth ± Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb</td>
<td>6</td>
<td>613</td>
<td>23.5 ± 9.7</td>
</tr>
<tr>
<td>Rb-C706F1</td>
<td>6</td>
<td>593</td>
<td>10.3 ± 6.2</td>
</tr>
</tbody>
</table>

Notes on Table 6: Quiescent VSMCs were microinjected with recombinant protein as described in the Materials and Methods section, then immediately stimulated for 24 hours with 10% FBS in medium containing BrdU. Afterwards, the cells were fixed and labeled for nuclear BrdU incorporation, and the percentage inhibition for each experiment was determined. For these experiments, BrdU labeling of quiescent VSMCs was 10.1 ± 1.2% (N=12, total number of cells counted = 2659); for uninjected FBS-stimulated VSMCs, 54.8 ± 2.4% (N =27, total number of cells counted = 4282); and for sham-injected FBS-stimulated cells, 49.6 ± 2.5% (N=27, total number of cells injected = 3401).

TABLE 7: Effect of Microinjected Rb Proteins on BALBc3T3 Cell Proliferation

<table>
<thead>
<tr>
<th>Protein Injected</th>
<th>Number of Experiments</th>
<th>Total Number of Cells Injected</th>
<th>Mean % Inhibition of FBS-stimulated Growth ± Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb</td>
<td>2</td>
<td>436</td>
<td>18.3 ± 8.0</td>
</tr>
<tr>
<td>Rb-C706F1</td>
<td>2</td>
<td>370</td>
<td>-9.4 ± 7.3</td>
</tr>
</tbody>
</table>

Notes on Table 7: Quiescent BALBc3T3 cells at approximately 75% confluence were microinjected with recombinant protein as described in the Materials and Methods section, then immediately stimulated for 24 hours with 10% FBS in medium containing BrdU. Afterwards, the cells were fixed and labeled for nuclear BrdU incorporation, and the percentage inhibition for each experiment was determined. For these experiments, BrdU labeling of quiescent BALBc3T3 cells was 5.8 ± 3.1% (N=5, total number of cells injected = 658); for uninjected FBS-stimulated cells, 64.3 ± 3.2% (N =9, total number of cells injected = 1364); and for sham-injected FBS-stimulated cells, 58.1 ± 3.9% (N=9, total number of cells injected = 1150).
Effect of Coinjected Id Protein on VSMC Growth Inhibition by MyoD

Id is known to encode a protein that is a dominant negative inhibitor of HLH transcription factor action. Consequently, we wished to test its ability to inhibit the action of MyoD protein in VSMCs. Only two experiments were attempted. Microinjection of Id protein appeared to cause VSMC death, as large numbers of ghosts were seen in the coverslip areas where VSMCs were microinjected with GST-Id protein, whether MyoD was coinjected or not (not shown). Thus, the results of these two experiments were inconclusive, especially since the GST-Id fusion protein was provided in phosphate buffered saline rather than a hypotonic solution, which might have harmed the cells in and of itself. However, again for completeness, the data from these experiments are summarized below in Table 8.

The reverse experiment was also done; i.e., we tested to see if microinjected Id was capable of inducing S-phase entry in VSMCs under serum-free conditions. Thus

<table>
<thead>
<tr>
<th>Protein Injected</th>
<th>Number of Experiments</th>
<th>Total Number of Cells Injected</th>
<th>Mean % Mitotic Index (FBS-stimulated Growth ± Standard Error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
<td>243</td>
<td>44.5 ± 3.0</td>
</tr>
<tr>
<td>Buffer</td>
<td>2</td>
<td>97</td>
<td>42.5 ± 2.0</td>
</tr>
<tr>
<td>GST</td>
<td>2</td>
<td>170</td>
<td>48.5 ± 6.9</td>
</tr>
<tr>
<td>Id*</td>
<td>2</td>
<td>161</td>
<td>32.2 ± 7.1</td>
</tr>
<tr>
<td>MyoD**</td>
<td>2</td>
<td>251</td>
<td>20.5 ± 9.1</td>
</tr>
<tr>
<td>MyoD and Id</td>
<td>2</td>
<td>181</td>
<td>26.5 ± 0.1</td>
</tr>
</tbody>
</table>

* Id was microinjected at a concentration of 0.5 mg/ml in all experiments.

** MyoD was microinjected at a concentration of 1 mg/ml in these experiments.
TABLE 9: Effect of Microinjected and Coinjected Id Protein on S Phase Entry in Quiescent VSMCs

<table>
<thead>
<tr>
<th>Protein Injected</th>
<th>Number of Experiments</th>
<th>Total Number of Cells Injected</th>
<th>Mean % Mitotic Index (FBS-stimulated Growth ± Standard Error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
<td>212</td>
<td>9.5 ± 0.9</td>
</tr>
<tr>
<td>Buffer</td>
<td>1</td>
<td>94</td>
<td>12.8</td>
</tr>
<tr>
<td>GST</td>
<td>2</td>
<td>171</td>
<td>16.8 ± 3.7</td>
</tr>
<tr>
<td>Id*</td>
<td>2</td>
<td>105</td>
<td>28.7 ± 16.9</td>
</tr>
<tr>
<td>MyoD**</td>
<td>2</td>
<td>153</td>
<td>18.9 ± 6.2</td>
</tr>
<tr>
<td>MyoD and Id</td>
<td>2</td>
<td>175</td>
<td>28.7 ± 7.8</td>
</tr>
</tbody>
</table>

* Id was microinjected at a concentration of 0.5 mg/ml in all experiments.

** MyoD was microinjected at a concentration of 1 mg/ml in these experiments.

Quiescent VSMCs were injected with Id protein, then labeled in serum-free medium containing BrdU and the percentage of labeled nuclei determined. These data are summarized in Table 9.

**Effect of Microinjected MHex on S Phase Entry in VSMCs**

Because MHex has been postulated to interact with the serum response factor to enhance its binding to the serum response element (see Chapter 1) and because its transcript has been shown to be serum-inducible (see Chapter 5), we wished to test whether or not microinjected MHex protein was able to cause quiescent VSMCs to enter S phase. Microinjected MHex appeared able to induce VSMC and BALBc3T3 cell entry into S phase very weakly (only approximately two-fold), and it is not clear whether this effect is statistically significant. If this effect is significant, it is clearly not very potent. The results of these experiments are summarized in Tables 10 and 11.
TABLE 10: Effect of Microinjected MHox on S Phase Entry in Quiescent VSMCs

<table>
<thead>
<tr>
<th>Protein Injected</th>
<th>Number of Experiments</th>
<th>Total Number of Cells Injected</th>
<th>Mean % Mitotic Index (FBS-stimulated Growth ± Standard Error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10</td>
<td>2942</td>
<td>11.0 ± 1.5</td>
</tr>
<tr>
<td>Buffer</td>
<td>10</td>
<td>2324</td>
<td>11.0 ± 4.4</td>
</tr>
<tr>
<td>Gax</td>
<td>4</td>
<td>708</td>
<td>9.5 ± 1.0</td>
</tr>
<tr>
<td>MHox</td>
<td>6</td>
<td>1216</td>
<td>21.5 ± 4.6</td>
</tr>
<tr>
<td>Ras[Leu-61] mutant</td>
<td>6</td>
<td>1387</td>
<td>40.0 ± 5.6</td>
</tr>
<tr>
<td>Rb-C706F</td>
<td>1</td>
<td>79</td>
<td>13.9</td>
</tr>
<tr>
<td>YY1</td>
<td>4</td>
<td>721</td>
<td>19.8 ± 6.3</td>
</tr>
</tbody>
</table>

Notes: Protein concentrations were as follows: Gax, 0.6 mg/ml; MHox, 1.5 mg/ml; Ras[Leu-61], 0.5 mg/ml; Rb-C706F, 1.6 mg/ml; YY1, 2 mg/ml.

TABLE 11: Effect of Microinjected MHox on S Phase Entry in Quiescent BALBc3T3 Cells

<table>
<thead>
<tr>
<th>Protein Injected</th>
<th>Number of Experiments</th>
<th>Total Number of Cells Injected</th>
<th>Mean % Mitotic Index (FBS-stimulated Growth ± Standard Error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>773</td>
<td>7.7 ± 1.6</td>
</tr>
<tr>
<td>Buffer</td>
<td>5</td>
<td>659</td>
<td>6.6 ± 1.2</td>
</tr>
<tr>
<td>Gax</td>
<td>1</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>MHox</td>
<td>5</td>
<td>611</td>
<td>12.5 ± 5.3</td>
</tr>
<tr>
<td>Ras[Leu-61] mutant</td>
<td>2</td>
<td>247</td>
<td>31.7 ± 12.0</td>
</tr>
<tr>
<td>Rb-C706F</td>
<td>1</td>
<td>108</td>
<td>22.2</td>
</tr>
<tr>
<td>YY1</td>
<td>4</td>
<td>373</td>
<td>9.4 ± 4.2</td>
</tr>
</tbody>
</table>

Notes: Protein concentrations were as follows: Gax, 0.6 mg/ml; MHox, 1.5 mg/ml; Ras[Leu-61], 0.5 mg/ml; Rb-C706F, 1.6 mg/ml; YY1, 2 mg/ml.
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