MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF PAIRED-LIKE HOMEODOMAIN TRANSCRIPTION FACTORS, Arix/Phox2a AND NBPhox/Phox2b, ON THE DOPAMINE ß-HYDROXYLASE PROMOTER

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TABLE OF CONTENTS

List of Tables and Figures

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ABSTRACT

The homeodomain transcription factors Arix/Phox2a and NBPhox/Phox2b play a critical role in the specification of noradrenergic neurons by inducing the expression of dopamine ß-hydroxylase (DBH), the terminal enzyme for noradrenaline biosynthesis. In the first part of the thesis, I have characterized NBPhox in comparison to Arix with respect to gene structure, chromosomal location, and transcriptional regulatory properties. Chromosomal location of NBPhox differs from that of Arix. NBPhox is located on human Chromosome 4p12 and mouse Chromosome 5. Yet, the gene structure is very similar, encompassing approximately 5kb. Both Arix and NBPhox are composed of three exons and two introns with an identical split between exon 2 and 3. Both proteins similarly bind to three homeodomain recognition sites in the proximal promoter of the rat DBH. They exhibit cooperative binding to the DB1 regulatory element which contains two homeodomain recognition sites. The cooperative binding may result from homo-dimerization of Arix and NBPhox since they undergo protein-protein interaction *in vitro*. The two transcription factors activate transcription from the rat DBH promoter with similar efficacies. In addition, Arix and NBPhox can synergistically augment DBH transcription together with simultaneous stimulation of the PKA pathway. Furthermore, the transactivation domain of Arix is mapped to the N-terminal segment, which shares 50% identity with NBPhox at the amino acid level, suggesting a similar mechanism in activating the DBH gene transcription. Thus, Arix and NBPhox regulate DBH gene transcription in an indistinguishable and independent manner.

In the second part of the thesis, I have evaluated whether post-translational modification of Arix regulates PKA-mediated DBH gene transcription. Arix is a

constitutive phospho-protein under basal conditions *in vivo*. Stimulation of the PKA pathway substantially decreases the phosphorylation state of Arix. The change in Arix phosphorylation state is paralleled with its DNA-binding activity. When cells are treated with forskolin, DNA-binding activity of Arix is significantly enhanced. This enhancement is reversed by treatment of cells with tyrosine and serine/threonine phosphatase inhibitors prior to forskolin stimulation. Consistent with the DNA-binding activity of Arix, the DBH promoter activity mediated by Arix and PKA is reduced by 40% by pretreatment of cells tyrosine phosphatase inhibitor. Taken together, these results suggest that dephosphorylation of Arix is a necessary step to fully activate PKA-stimulated DBH transcription. The functional phosphorylation sites appear to locate in either the homeodomain or the N-terminal segment of Arix. Thus, the studies in the second part of this thesis demonstrate that post-translational modification of Arix can regulate the DBH gene transcription in response to activation of the PKA pathway.

Chapter One

Introduction

Catecholamine Biosynthesis

Catecholamines are a group of neurotransmitters and hormones in the central and peripheral nervous systems (CNS and PNS, respectively), consisting of dopamine, noradrenaline, and adrenaline. These substances are released from neurons and endocrine cells. They participate in a wide range of behaviors, physiological mechanisms, drug actions, and neurological, psychiatric, endocrine, and cardiovascular diseases. Adrenaline and noradrenaline are major effector biochemicals used in the body's stress system, often referred to as "the fight and flight" response. On the other hand, dopamine, a major constituent of the brain catecholamines, is synthesized in many areas of the CNS. In particular, dopamine produced from the substantia nigra is transmitted to the striatum, which controls motor functions. Intensive investigation of catecholamines have resulted in an enormous body of knowledge over the past several decades, contributing to a variety of clinical aspects. This thesis further investigate catecholamines with regard to the regulation of the noradrenaline biosynthetic enzyme, dopamine ß-hydroxylase (DBH).

The biosynthesis of catecholamines occurs in several steps as shown in Fig 1. All substances are synthesized from the same precursor, tyrosine. The first step is the hydroxylation of tyrosine, catalyzed by tyrosine hydroxylase (TH), generating DOPA, 3,4-dihydroxyl-phenylalanine (107). In addition to molecular oxygen, this reaction requires tetrahydrobiopterin (BH_4) as a cofactor, which donates a hydrogen atom to form a new hydroxyl group (13, 141). The second step involves the formation of dopamine, which results from decarboxylation of DOPA by DOPA-decarboxylase (DDC) (15). Once dopamine is synthesized, it will be taken up to either vesicles in neurons or granules

in chromaffin cells via a vesicular membrane transporter (VMAT) (29, 87). Subsequent production of noradrenaline occurs in the vesicles. In the third step, dopamine ß-hydroxylase (DBH) converts DOPA to noradrenaline (22). DBH uses molecular oxygen to form the hydroxyl group and ascorbic acid as a source of electrons (25). In addition, Cu^{2+} is necessary for the enzymatic activity of DBH and is involved in electron transfer in the reaction [(33), reviewed by (146)]. The last step is concluded by phenylethanolamine N-methyltransferase (PNMT). Noradrenaline, which is produced in the vesicles, is transported back to the cytoplasm and becomes a substrate for PNMT (21). PNMT transfers a methyl group from S-adenosylmethionine to the nitrogen of noradrenaline, generating adrenaline (18). The catecholamines are then packaged in vesicles or granules via VMAT for storage and subsequent release (29, 87). Studies with knock out mice clearly indicate the importance of the enzymes involved in the catecholamine biosynthetic pathway since the targeted gene deletion of TH (175) and DBH (154) results in loss of viability.

The rate of catecholamine biosynthesis is regulated by TH, presumably because the maximum velocity of TH is slower than other enzymes in the biosynthesis pathway. Consequently, TH governs the over all rate of synthesis of catecholamines. It is worthwhile to note that TH is thought to be at the level of about 70-80% saturation with the substrate, tyrosine, under the resting state. Therefore, the availability of BH_4, the cofactor of TH, may be essential for catecholamine synthesis *in vivo* and may, in fact, be the rate-limiting factor. Indeed, failures in the biosynthetic pathway as well as recycling of BH_4 lead to a decrease in TH activity (66). This leads to severe deficits in

catecholamines, causing developmental delay and progressive neurological deterioration with symptoms of parkinsonism.

Since TH is the rate-limiting enzyme of catecholamine synthesis, many studies were carried out to understand its enzymatic regulation. Early studies on TH regulation indicated end-product inhibition of its enzymatic activity [reviewed by (94)]. Free intraneuronal catecholamines inhibit the further activity of TH by competing for the BH_4 binding site (102). Conversely, upon depolarization, when cytoplasmic concentrations of catecholamines are lowered by release from nerve terminals upon depolarization, disinhibition of TH occurs (103, 127). This effect appears to be mediated by phosphorylation in the regulatory domain of TH by PKA, CaMKII, and MAP kinase (49, 50, 169, 177). This phosphorylation likely to shift the conformation of TH to a catalytically active form. In addition to the rapid regulatory mechanisms described above, long-term regulation of catecholamine synthesis occurs at transcriptional levels of both TH and DBH. When the neuronal activity is increased for a prolonged period, mRNA levels for TH and DBH are elevated, eliciting an increase in newly synthesized enzymes (10, 152, 176). Transcriptional regulation of DBH will be described more extensively in the later section of this chapter.

Cellular compartmentalization and tissue distribution of the enzymes involved in catecholamine synthesis are distinct. TH is found in all cells that synthesize catecholamines. It is primarily localized in the cytoplasm, but is also in the plasma membrane (52, 76). The second enzyme, DDC, lacks tissue specificity. DDC is a non-specific amino acid decarboxylase present in neurons as well as in non-neuronal tissues such as the liver, stomach, kidneys and blood vessels (6). Although DDC is the last

enzyme to produce dopamine in dopaminergic neurons, the expression of TH characterizes dopaminergic neurons. The third step of catecholamine synthesis occurs in synaptic vesicles for noradrenergic neurons and in chromaffin granules for adrenal medulla, since DBH is concentrated in those compartments [reviewed by (166)]. Most of DBH is bound to the inner vesicular membrane, while some is soluble within the vesicles [(129, 133), reviewed by(146)]. To represent noradrenergic neurons, coexpression of TH and DBH is necessary. PNMT is a soluble protein in the cytoplasm, found in both the adrenal medulla (18) and in a small population of neurons in the brainstem (38, 83). Expression of PNMT is a distinguishing feature of adrenergic cells. Thus, combinatorial expression patterns of the enzymes for catecholamine synthesis determine three neurotransmitter phenotypes: dopaminergic, noradrenergic, and adrenergic neurons.

Physiological Roles of Noradrenaline

Noradrenaline is a neurotransmitter in the brain as well as in postganglionic sympathetic neurons. Noradrenergic cells are found in the locus ceruleus (LC), which is the center of noradrenergic neurons in the brain. Even though the LC comprises a very small population, only about 1600 cells on each side of the rat brain (126), its axons demonstrate broad innervation: dorsally to the cerebellum, caudally to the spinal cord, and rostrally to virtually all regions of the telencephalon and diencephalon, including the neocortex, hippocampus, amygdala, spetum, thalamus, and hypothalamus (86). Thus, LC modulates a variety of important behavioral and physiological processes by providing noradrenergic input to the target regions. Indeed, electrophysiological and behavioral studies have shown that firing of LC neurons reflects changes in behavioral activity and sensory input such as attention, arousal, and vigilance. Arousing sensory stimuli activate LC neurons (5), while the performance of maintenance functions, such as sleeping, grooming, and ingestive behaviors, inhibit LC neurons (4). Based on these findings, Aston-Jones hypothesized that noradrenaline mediates an organism's level of vigilance; that is, attention and behavioral reactivity to salient external stimuli (3).

Another role of noradrenaline is regulation of hunger and feeding behavior. Noradrenaline in the paraventicular nucleus of the hypothalamus, one of terminal areas receiving projection from the LC, elicits robust eating response, even in previously satiated animals (82). Furthermore, neuropeptide Y, which often colocalizes with noradrenaline in many noradrenergic fibers, potentiates the influence of noradrenaline on feeding behavior [reviewed by (81)]. Interestingly, noradrenaline and neuropeptide Y levels in the paraventicular nucleus exhibit a circadian rhythm with a peak at the

beginning of the dark phase of the light-dark cycle, during which most food intake occurs (153).

Recent studies using mice specifically lacking the ability to synthesize noradrenaline have clearly demonstrated that noradrenaline is essential for normal development, physiology and behavior. Researchers lead by Palmiter successfully created noradrenaline deficient mice by disrupting the DBH locus (154). Most of these *Dbh -/-* mice die *in utero* at about E11.5, apparently due to cardiovascular failure as examined by histological phenotype. However, mutant embryos can be rescued by perinatal administration of either dihydroxyphenylserine, a direct precursor of noradrenaline, or isoproterenol, a ß-adrenergic receptor agonist. Noradrenaline-deficient mice have impaired adaptation to cold and elevated basal metabolic rates without abnormalities in thyroid hormone levels. This suggests that noradrenaline plays a fundamental role in determining the basal metabolic rate independent of thyroid activity (154). Furthermore, the noradrenaline-deficient mice are more easily sedated by alcohol (162) and have an increased susceptibility to seizure-inducing agents (151). Reproduction is also impaired in these mice; males are infertile and females have defects in nurturing behavior (155). Thus, the genetic approach using noradrenaline-deficient mice has proven to be interesting and useful to understand the physiological role of noradrenaline in a variety of aspects.

In the PNS, noradrenergic cells are found in sympathetic ganglia and in the chromaffin cells of the adrenal medulla. The sympathetic nervous system is a component of the autonomic nervous system that modulates the functions of internal organs in order to achieve homeostasis. Sympathetic neurons innervate virtually all visceral organ,

including eye, heart, respiratory tract, stomach, small intestine, colon, kidney, blood vessel, and sweat glands. Thus, the functions of these organs are affected by noradrenaline input either by stimulating or by inhibiting them. For example, excitement or stress activates the sympathetic nervous system. As a result, release of noradrenaline increases heart rate and respiration and inhibits gastrointestinal activity. Actions of noradrenaline go through α- and/or ß-adrenergic receptors, activating distinct second messenger pathways depending on a receptor subtype [reviewed by (27, 119, 174)]. Receptor subtype-selective agonists and antagonists are widely used for clinical treatment, such as hypertension, asthma, and various cardiac conditions.

With respect to the clinical relevance of DBH, a small minority of patients with autonomic failure have been reported to be DBH deficient (125). This is characterized by complete absence of the DBH enzyme and a rare, congenital, and nonhereditary disorder. These patients exhibit elevated plasma dopamine concentration as well as undetectable noradrenaline and adrenaline concentrations. The major clinical presentations in the DBH-deficient patients are orthostatic hypotension and impaired exercise tolerance. According to the recent report by Kim *et al.*, the genetic cause of DBH deficiency is mutations in the DBH locus, creating a non-sense protein; one mutation was found in the splice donor site of *DBH* intron1; another in *DBH* exon2 (71). Given the fact that Dbh -/- mice result in fetal lethality, it is surprising that the patients can survive and can be treated with dihydroxylphenylserine. It is, therefore, reasonable to predict that humans have a compensatory mechanism to overcome the deficit of noradrenaline.

Transcriptional Regulation of DBH

Among the catecholamine-synthesizing enzymes, TH was the first enzyme whose cDNA was cloned. A full-length cDNA containing the entire coding sequence of TH was cloned from rat pheochromocytoma by Grima *et al.*, in 1985 (44). During the next 5 years, many groups spent a great effort to isolate genes for all catecholamine-synthesizing enzymes, TH, DDC, DBH, and PNMT. Multiple mRNA transcripts have been found from human TH, DBH, and PNMT genes while DDC gene has only one known transcript [reviewed by (106)]. Five'-upstream promoter regulatory sequences for these genes have been isolated as well. Isolation of cDNAs and genomic DNAs for catecholamine-synthesizing enzymes has certainly accelerated the understanding of their regulation.

Human DBH has two types of mRNA transcripts. They are derived from a single gene, but through a different mechanism: alternative polyadenylation. Both transcripts of DBH encode the same amino acid sequence, but differ in the 3'-untranslated region. Distribution of multiple mRNA transcripts is dominated by one form of mRNA in the adrenal medulla. Interestingly, both soluble and membrane-bound forms of DBH were produced from one primary translation product, which is sufficient to exhibit enzymatic activity (84). The functional significance of the production of multiple mRNAs with different 3'-untranslated regions is still unknown. One possibility is that the 3'-untranslated region is involved in the stability of mRNA and translational efficiency. Human TH and PNMT are also encoded by a single gene. However, the multiple mRNA transcripts are produced by different mechanisms: an alternative splicing mechanism for TH and an alternative promoter mechanism for PNMT (106).

In the late 1980s, researchers observed the elevated mRNA level of TH in the adrenal gland, sympathetic ganglion, and the brain in response to a variety of stimuli, including dexamethasone (85) as well as cold and immobilization stress (8, 10, 124). In addition, the elevation of TH mRNA was accompanied with parallel increases in TH enzymatic activity, suggesting the transcriptional regulation of TH activity. DBH and PNMT also revealed transcriptional regulation. Since then, studies on the transcriptional regulation of catecholamine-synthesizing enzymes have extensively carried out. We have begun to understand that the transcriptional regulation is the more important mechanism for regulating long-term enzymatic activity in response to drug treatments or a variety of stress [reviewed by (130)].

Although TH receives more attention as a key regulator of overall catecholamine synthesis, we can not ignore the long-term regulation of DBH which is necessary for noradrenaline production. In *in vivo* systems, administration of glucocorticoids (109), repeated immobilization stress (108), and chronic treatment with reserpine (167) are known to steadily raise the mRNA levels of DBH in noradrenergic tissues including the adrenal medulla, sympathetic ganglia, and locus ceruleus. The increases in DBH mRNA are steady over a long period and accompanied with increased enzymatic activity of DBH (58, 72, 167).

In order to understand the transcriptional regulation of DBH, our laboratory and others have extensively investigated the 5'-upstream promoter regulatory sequence of DBH in the past several years. Initially, Shaskus *et al.*, isolated a clone from a rat genomic library that contained 395 bases of 5'-upstream sequence from the transcription initiation site and 200 bases of transcribed sequence (139). A segment containing the 395

bases of 5'-flanking start site was then fused to chloramphenicol acetyltransferase reporter plasmid (DBH-CAT/-395) for further promoter analyses. This promoter construct, DBH-CAT/-395 was sufficient to drive cell-type specific regulation: promoter activity was greater in neuronal cell lines expressing endogenous DBH including SHSY-5Y neuroblastoma, and PC12 pheochromocytoma cell lines than in DBH-negative cell lines such as CV-1 monkey kidney, JEG-3 choriocarcinoma, and C6 glioma cell lines. Within the 395 base fragment, several sites homology to the known regulatory elements were found, as depicted in Fig. 2. These elements are activator protein 2 (AP2), human cyclic responsive element homolog (hCREh), CRE/AP1, and homeodomain protein recognition 1, 2, and 3 (HD1, 2, and 3) sites. In addition, Yin and Yang 1 (YY1) and SP1 sites have been found only in the human DBH promoter (137). Among them, AP2, CRE/AP1, HD1, 2, and 3, and YY1 sites have been identified as functional elements to regulate the DBH promoter and will be discussed next. The functional importance of the HD1, 2, and 3 sites will be described in the following section, "Arix/Phox2a and NBPhox/Phox2b," of this chapter.

Treatment of cells with forskolin or phorbol ester, which activates the PKA or PKC pathway, respectively, results in an enhancement of DBH reporter gene activity (139). The simultaneous stimulation of both effectors elicits a synergistic increase in the DBH promoter activity. The endogenous mRNA level of DBH is also consistently increased upon activation of the PKA and/or PKC pathways. In order to identify the elements that mediate DBH promoter activation by PKA and PKC, progressive deletion analyses were performed and identified a 30 base genetic regulatory element, designated DB1 (139). The DB1 fragment is an enhancer that carries two important features: cell-

type specificity and second messenger responsiveness. DB1 is thought to be a transcriptional hot spot because of the presence of multiple regulatory elements, including hCREh, CRE/AP1, YY1, and HD1/2. A hetelogous reporter construct composed of two copies of DB1 followed by thymidine kinase promoter enables to drive transcription in only catecholaminergic cell lines.

To further refine the elements responsible for PKA responsiveness on the DBH transcription, promoter analyses were carried out using mutant DBH-reporter constructs. PKA responsiveness of DBH transcription is mapped to the CRE/AP1 site in the DB1 (150). Electrophoretic mobility shift assay (EMSA) consistently demonstrated the formation of a cAMP-induced complex that binds to the DB1 in the CRE/AP1 site dependent manner. Furthermore, cFos and cJun are identified from PC12 nuclear extracts as components of the cAMP-induced complex on the DB1 (149). The functional importance of cFos and cJun complex recruitment is further supported by the transfection of a dominant negative JunD expression plasmid in PC12 cells. This results in partial inhibition of the enhancement of DBH promoter activity by PKA. Thus, PKA-stimulated DBH transcription is partly mediated by recruiting cFos and cJun to the CRE/AP1 site.

Seo *et al.* have reported another transcription factor binding site, a YY1 element, on the human homolog of DB1. The YY1 element, binding of the ubiquitous transcription factor YY1, bridges over hCREh and CRE/AP1 sites. Results from EMSA suggest the binding of YY1 factor to DB1 using nuclear extracts from DBH-positive neuroblastoma, SK-N-BE(2)C cells, but not from DBH-negative HeLa cells (137). A single nucleotide mutation at the YY1 site, which does not disrupt either hCREh or CRE/AP1 sites, leads 70% reduction in the basal DBH promoter activity. Interestingly,

the mutation at the YY1 results in overall decrease in cAMP-induced DBH transcription, yet enhances cAMP inducibility. Collectively these results suggest that the YY1 element positively regulates basal transcription of DBH in a cell-type specific manner, while simultaneously, negatively controlling cAMP-mediated induction.

We should also consider the role of transcription factor, AP2, in the DBH transcription. An AP2 binding site was identified on the rat DBH promoter by DNase I footprint analyses using recombinant AP2 protein (43). It encompasses bases –136 to –115 of the rat DBH promoter, bearing a near-consensus binding site for AP2 (GCCNNNGGC) (164). Disruption of the AP2 site severely decreases the basal DBH promoter activity as well as the responsiveness of the DBH promoter to cAMP by 50%. In addition, exogenous expression of the AP2 protein enhances basal DBH promoter activity in HepG2 cells, which have a low expression level of AP2. Thus, the AP2 element is necessary to maintain the basal promoter activity of the DBH gene and to partially mediate the second messenger responsiveness. Although AP2 is expressed predominantly in neural crest cell lineage including sympathetic ganglia (101), whether the AP2 site acts in tissue-specific manner has yet to be determined.

The DBH gene expression is also under negative control to inhibit the noradrenergic phenotype in inappropriate cells. Detailed analyses of genetic regulatory elements using progressive deletion mutation of the 5'-flanking region of the DBH gene identified a fragment between –394 and –232 that represses transcription from the DBH promoter in non-catecholaminergic cell lines (140). Further analyses refined the location of a putative DBH negative regulatory element, DBH-NRE, between –282 and –232. DBH-NRE exhibited a stronger repression activity to a minimal TH promoter in non-

catecholaminergic cells than in catecholaminergic cells, suggesting that DBH-NRE may contribute to restrict the expression of DBH in inappropriate types of cells. Despite the cell-type specific character of DBH-NRE, nuclear factors bound to DBH-NRE are apparently present not only in catecholaminergic cells but in non-catecholaminergic cells.

A fragment of the human DBH promoter between - 403 and - 382 bp was a potential candidate element to repress the DBH transcription in non-neuronal cells because of its high homology to the restrictive element 1 (RE1) / neuron-restrictive silencer element (NRE) (70). RE1/NRE has been known to repress several neuronal genes, including SCG10 and typeII sodium channel genes, but only in non-neuronal cells. It has been proposed to mediate the repression of neuronal specific genes in non-neuronal cells through the binding of a zinc-finger protein, RE1-silencing transcription factor (REST) / neuron-restrictive silencer factor (NRSF) [reviewed by (64, 135)]. However, it is puzzling that the RE1/NRE-like element of the DBH gene exerts negative regulation similarly in both DBH-positive and negative cell lines and that the forced expression of REST / NRSF did not suppress the DBH promoter activity (70). Thus, the RE1/NRE-like element of the DBH gene is less likely to function as an exclusive silencer of DBH in non-neuronal specific pattern.

Kim *et al.* have proposed two other putative silencer regions upstream of the human DBH gene, SI and SIII, residing at -217 to -250bp and at -348 to -324bp, respectively (70). SI and SIII demonstrate nuclear protein binding activities from DBH-negative cell lines in a cell-type specific manner by DNaseI foot printing assays. Furthermore, SI and SIII diminished the heterologous thymidine kinase and homologous DBH proximal promoter activities preferentially in non-neuronal cell lines. These

findings suggest that silencing the DBH gene in inappropriate cells may involve multiple cis-regulatory elements located upstream of the DBH gene. It is, however, an enigma that none of tissue-specific nuclear factors that mediate the repression of DBH expression through silencer elements has been identified to date. It is still open to question how the DBH transcription is negatively regulated in non-neuronal cells.

It is also important to evaluate the activity of the promoter in *in vivo* . Using transgenic mice, a 5.8kb fragment of the human DBH gene upstream from the transcription start site has been shown to drive the expression of a reporter gene in the noradrenergic and adrenergic cells of the peripheral sympathetic nervous system, adrenal medulla and brain (100). Kobayashi *et al.*, also demonstrated that a 4kb fragment of the 5'-flanking region of the human DBH gene achieves the tissue-specific expression of DBH (74). In addition, the transgene products derived from the 4kb promoter are enzymatically active to produce norepinephrine. They exist in the secretory vesicles as both membrane-bound and soluble forms, similar to the subcellular localization of the normal DBH (74). However, in these transgenic mice, ectopic expression of the reporter gene or DBH transgene has been reported from the 5.8 and 4kb human DBH promoters in non-noradrenergic neurons, suggesting that these promoter fragments lack the negative regulatory elements, that restrict expression to the proper subset of neurons.

It should be noted that a 1.1kb fragment from the transcription start site is a minimal promoter that drives noradrenergic specific expression of the reporter gene in transgenic mice (56). A promoter fragment smaller than 0.6kb was unable to achieve the reporter gene expression in transgenic mice. These results are contradictory to those from the tissue-culture transfection assays, in which less than 0.6kb fragment can drive

reporter gene expression in catecholaminergic cell lines but not in non-neuronal cell lines (59, 79, 139). This may suggest that transgenic mice require more stringent conditions for tissue-specific expression than tissue-culture systems. Alternatively, we can not exclude the possibility that distal regulatory elements located between –0.6 and –1.1kb may block the function of proximal silencer elements located within the 0.6kb fragment; therefore, the 0.6kb promoter fragment was unable to achieve the reporter gene expression in transgenic mice. Coordinated regulation between distal and proximal regulatory elements may be necessary for the appropriate expression of DBH, as this type of regulation has been observed in other genes (42, 120).

Arix/Phox2a and NBPhox/Phox2b

In seeking the identity of proteins that direct tissue-specific expression of DBH, our laboratory initially cloned a cDNA encoding a homeodomain protein, that interacts with the DB1 enhancer element of the rat DBH gene (173). This cDNA was isolated from a PC12 expression cDNA library and named Arix for the extensive homology to *Drosophila aristaless* within the homeodomain. As detected by northern blots and RNA protection assays, Arix mRNA transcripts are present only in sympathetic ganglia, adrenal medulla, locus ceruleus and DBH-positive catecholaminergic cell lines, exhibiting the distribution specific to noradrenergic tissues and cell lines.

Independently, a mouse homologue of Arix was isolated by Valarche *et al* (159). It was named Phox2a due to the high homology to the mouse Phox1 homeodomain. Phox2a was identified in an attempt to identify transcription factors specific to the neuronal cell adhesion molecule (NCAM) expression. Valarche *et al.* screened the expression library from a neural crest derived neuroblastoma cell line, N2a cell, using a probe containing two consecutive homeodomain recognition sites from the NCAM promoter. In their screening, another cDNA encoding Cux/CDP was isolated in addition to Phox2a. Cux/CDP is also a homeodomain protein and appears to acts as a repressor [reviewed by (110)]. The recombinant proteins expressing the homeodomain of Phox2a and Cux/CDP were able to bind to the NCAM promoter in EMSA. In neuroblastoma N2a cells, NCAM promoter was further activated by transfecting in Phox2a expression plasmids. In contrast, Cux/CDP downregulates the NCAM promoter activity in N2a cells. This inhibitory effect is relieved by simultaneous over-expression of Phox2a. Since Cux/CDP is ubiquitously expressed, it is possible that Cux/CDP plays a role in

restricting the DBH expression in non-neuronal cells where Phox2a protein is absent. At present, it remains unknown whether Cux/CDP can directly or indirectly modulate the DBH promoter.

After isolation of Arix/Phox2a, a closely related Arix/Phox2a homeoprotein was identified and named NBPhox and Phox2b, from human and mouse, respectively (1, 171, 172). Arix/Phox2a and NBPhox/Phox2b are closely related homeodomain proteins and share high similarity at the amino acid level, bearing an identical homeodomain (Fig1.3A, B). Within the homeodomain, extensive homology is observed from paired and paired-like classes of homeodomain proteins, including Phox1, Aristaless, Alx3, Smox3, Paird, and Pax7 (Fig1.3C). Arix and NBPhox belong to the paired-like class of homeodomain proteins due to the presence of a glutamine rather than a serine residue at position 50 of the third helix, the DNA recognition helix, of the homeodomain (35). In contrast, the paired class homeodomain proteins are characterized by the presence of a paired-domain, which is the second DNA binding motif. Arix and NBPhox lack the paired domain and their homeodomain is the only DNA-binding domain.

Detailed analyses of the expression patterns of Phox2a and Phox2b have prompted us to understand the role of these proteins during embryogenesis. They are mostly co-expressed exclusively in neurons of the CNS and PNS, that express DBH, the key enzyme for noradrenaline biosynthesis (116, 156). The expression of Phox2a/2b starts as early as E9.5 and persists into postnatal stages in most of the noradrenergic neurons (summarized in Fig1.4). Since Phox2a/2b expression proceeds that of DBH, these homeodomain proteins have been suggested as determinants of the noradrenergic neurons.

In support of the above notion, knock-out mice of Phox2a and Phox2b clearly demonstrated the necessity of both proteins for generation of noradrenergic neurons (summarized in Table 1.1). Inactivation of the Phox2a gene leads to the agenesis of parasympathetic ganglia as well as the locus ceruleus, the main noradrenergic center of the brain (104). These mice also display altered morphology of the superior cervical ganglion (SCG), a group of sympathetic neurons, and massive atrophy of cranial sensory ganglia. However, the SCG in Phox2a -/- mince continue to express DBH, while the cranial ganglia loses the DBH expression. All these affected tissues in Phox2a -/- mice normally express Phox2a as well as DBH in wild-type mice, suggesting that Phox2a is necessary for proper development of noradrenergic cells in both the CNS and PNS. One striking fact in Phox2a -/- mice is that some of Phox2a expressing neurons are severely affected, whereas others such as, SCG, though smaller than wild-type, continue to express DBH. A plausible explanation is the possibility of functional redundancy with another gene, such as Phox2b that may compensate for the loss of Phox2a in certain cells.

Extensive analyses of the relative timing of Phox2a and Phox2b expression at various stages of development demonstrated that the expression of Phox2a proceeds that of Phox2b in tissues that are affected by inactivation of Phox2a gene (116). For example, in the locus ceruleus, Phox2b expression is no longer detectable in the Phox2a deficient mice. In contrast, the expression of Phox2b is independent of that of Phox2a in tissues that are not affected in Phox2a -/- mice. In addition, expression of either Phox2a or Phox2b immediately follows that of the other, suggesting positive cross-regulation between the two genes.

Consistent with the evidence that the expression of Phox2a and Phox2b cross-regulate each other, a targeted gene deletion of Phox2b results in the failure to develop mature sympathetic and parasympathetic ganglia (117). In these tissues, Phox2b expression turns on before Phox2a. Mutant ganglionic analagen also fail to induce the genes of DBH, TH, and Phox2a. In the locus ceruleus of Phox2b -/- mice, the expression of Phox2a is transient. Phox2a is expressed in early development at E10.5 and becomes absent in later development at E18.5; therefore, the expression of TH and DBH are absent in Phox2a -/- mice (115). This observation suggests that Phox2b is required for maintenance of the expression of Phox2a. Thus, Phox2b not only induces the expression of Phox2a in the PNS, but also maintains it in the CNS. Collectively, these studies on knock-out mice imply that Phox2a/2b are essential for specification of noradrenergic phenotype, probably by activating DBH and TH genes.

Tissue-culture systems have shown more direct evidence that Arix/ Phox2a activates DBH and TH genes. In non-catecholaminergic cell lines, such as hepatoma HepG2 or teratocarcinoma F9 cells, which lack endogenous expression of Arix, forced expression of Arix substantially stimulates promoters of DBH and TH by 3-5 fold (150). Yang *et at.* also demonstrated direct activation of DBH by Phox2a and Phox2b, but not TH (170). When Arix is expressed together with activation of the cAMP/PKA pathway, DBH promoter activity is substantially increased (150). Potentiation of the DBH promoter by Arix and PKA is synergistic, in that the elevation of transcription is more than additive effect of Arix and PKA alone. In contrast, Arix and PKA act independently on the TH promoter, eliciting an increase in the TH promoter activity, which is approximately the sum of reporter activity by Arix and PKA alone.

Three elements on the DBH promoter which are involved in Arix's action, have been identified. These are homeodomain recognition (HD) 1, 2, and 3 site as shown in Fig.1.2. The HD1 and 2 sites are separated by 6 bases and are located adjacent to the CRE/AP1 site within DB1. The HD3 site, on the other hand, resides closer to the TATA-box. The promoter proximal HD3 site participates in the basal DBH promoter activity (148), while the distal HD1/2 sites are necessary for the maximal response to PKA-stimulated DBH transcription. The effectiveness of the HD1/2 sites depends on the presence of the intact HD3 site. Thus, these HD sites are functionally interdependent and synergistically maximally activate the DBH promoter. Similarly, Kim *et al.* reported that domain II in the human DBH promoter located between –56 and –87bp, the area which contains the HD3 site, is responsive to Phox2a (69). Interestingly, domain II has a strong noradrenergic specificity. An artificial reporter construct composed of four tandem copies of domain II strikingly increases reporter activity in DBH-positive cell lines. Moreover, forced expression of Phox2a in DBH-negative cell lines confers the artificial reporter construct as active as in DBH-positive cell lines. Thus, the HD3 site appears to play a critical role in the DBH transcription by facilitating the basal promoter activity and by conferring tissue specificity.

The synergistic interaction of Arix and PKA in the activation of the DBH promoter may be an important event *in vivo*. Environmental signals encountered during the development of noradrenergic cells lead to the activation of intracellular second messenger pathways, such as the PKA pathway, which, in turn, interact with tissue-specific transcription factor, Arix, resulting in the potent expression of DBH and TH. This results in the specification of the noradrenergic neurotransmitter phenotype. This

idea is further supported by experiments using primary cultures of neural crest stem cells established from E10.5 rat embryos (88). Forced expression of Phox2a induces the expression of endogenous TH in neuronal crest culture treated with forskolin, an activator of adenyl cyclase, which activates the PKA pathway by an elevation of intracellular cAMP levels. However, this condition is not sufficient to drive the expression of DBH and pan-neuronal genes. When cells are cultured together with forskolin and bone morphogenic protein (BMP2), robust induction of the DBH expression is observed. This phenomenon is not observed with forskolin or BMP2 alone. Although BMP[1] is a natural environmental growth factor in embryo and is known to activate the expression of endogenous Phox2a, it is not sufficient to further activate the DBH expression in rat neural crest cultures (88). This suggests that Phox2a integrates the influence of multiple extracellular signals to coordinate the expression of subtype-specific genes for noradrenergic identity.

In addition to the studies using primary neural crest cultures, Stanke *et al.*, elegantly demonstrated sufficiency of Phox2a/2b to promote the development of noradrenergic neurons in the developing chick embryo (145). In their studies, overexpression of Phox2a/2b was achieved directly in chick embryos by implanting fibroblasts that express a viral expression vector containing Phox2a/2b cDNAs. In this experimental system, forced expression of Phox2a or Phox2b resulted in the ectopic generation of neurons that express the noradrenergic marker genes, TH and DBH, as well as pan-neuronal genes, SCG10 and NF160. Neural crest cultures have developmental potential, yet lack certain factors in their normal environment. Those factors may be

[1] Developmental connection of BMP and Phox2a/2b is described in the following section.

compensated in the developing chick embryo. Thus, it is likely that Phox2 proteins integrate cofactors present in the embryo, that presumably activate the PKA pathway to direct the noradrenergic phenotype by inducing the expression of DBH and TH.

Transcriptional Control of Noradrenergic Phenotype Differentiation

One of the challenging questions in developmental biology is to understand how the cellular diversity is generated in the nervous system. In the developing nervous system, cell fate specification is assigned through the interplay between extrinsic signals present in a cell's local environment and intrinsic signals that are governed by the cell's internal program [reviewed by(28)]. Ultimately, the identity of any cell is defined by a coordinated array of gene expression. In particular, the specification of the neurotransmitter phenotype is an important aspect of neuronal fate determination. Recent studies using knock-out mice, stem cell cultures, and chicken embryo cultures have begun to identify the crucial factors involved in controlling choice of the neurotransmitter [reviewed by (39)].

Neural crest cells have often been employed to study the specification of the noradrenergic phenotype since sympathetic ganglia, one of the major noradrenergic cells, is derived from neural crest cells. They are initially multipotent, can be isolated from the developing rat embryo for cultures and can be reconstituted with appropriate factors to differentiate into a certain lineage. During development, neural crest cells located at the most dorsal part of the neural tube detach and migrate along a number of defined routes to various tissues where they stop moving and differentiate into various cell types, such as sympathetic and sensory neurons, peripheral ganglia, smooth muscle, and melanocytes [reviewed by (2)]. The fate of neural crest cells can be manipulated by transplanting them into different locations or by exposing them to different environments *in vitro*. Together with studies from loss-of-function analyses in mice, three key players for

noradrenergic phenotype specification have been identified: BMP, mammalian acheate-scute homolog 1 (Mash1), and Phox2a/2b.

BMPs represent more than 20 members of the transforming growth factor-ß family and show a remarkably broad spectrum of functions during development [reviewed by (99)]. The first evidence for the involvement of BMP in noradrenergic induction was demonstrated by Varley *et al* (160). They observed an increased number of catecholamine-positive cells when avian neural crest cells were cultured in the presence of recombinant BMP7. This increase was paralleled by an increase in the number of TH-positive cells. Subsequently, the endogenous expression of BMPs were found in the dorsal aorta, which closely locates migrating sympathetic precursors (122). These observations implied that secreted BMPs from the dorsal aorta induce the noradrenergic differentiation of sympathetic precursors.

Mash1 is a basic helix-loop-helix transcription factor, cloned as a mammalian homologue of Drosophila Achaete-Scute (62). In Drosophila, achaete-scute are proneuronal genes required for the determination of neural fate in the ectoderm. In the rat embryo, Mash1 expression is confined to a subpopulation of neural precursor cells in the CNS and PNS, including forebrain, and sympathetic and enteric neurons (90). Similar to Drosophila Acheate-Scute, Mash1 expression is transient and extinguished before, or shortly, after overt neuronal differentiation. The inactivation of the Mash1 gene eliminates virtually all mature sympathetic and parasympathetic neurons as well as olfactory and retinal neurons (47). Thus, the above results suggeste that Mash1 is necessary for proper development of the autonomic nervous system, possibly by instructing precursors to become competent for lineage specific differentiation.

The link among BMP, Mash1 and Phox2a/2b has been established by several lines of evidence. First, exposure of rat neural crest cells to BMP2 rapidly induces the expression of Mash1, and subsequently that of Phox2a (89). Second, direct implantation of agarose beads coated with Noggin, a BMP antagonist, in the vicinity of the dorsal aorta of chick embryo, prevents generation of sympathetic neurons (134). Noggin-treated embryos also lose the expression of Cash1, a chicken homologue of Mash1, Phox2a/2b, TH, and DBH. Third, in Mash1 knock-out mice, the expression of Phox2a, as well as DBH and TH, is abolished (51). Conversely, in the absence of BMP, constitutive expression of Mash1 in neural crest cells induces expression of Phox2 and promotes morphological neuronal differentiation by expressing pan-neuronal markers (89). Collectively, the above evidence suggests that BMP triggers a genetic cascade from Mash1 to Phox2a/2b followed by subtype-specific genes, TH and DBH, and pan-neuronal genes.

As described in the previous section, the link between Phox2a/2b and the catecholamine biosynthesizing enzymes, TH and DBH, has been demonstrated both *in vivo* and *in vitro*. It should be emphasized that neither BMP treatment nor constitutive expression of Mash1 or Phox2a/2b are able to promote the expression of the subtype-specific genes, TH and DBH, in the above experiments. The induction of TH and DBH can be achieved by an elevation of intracellular cAMP along with BMP treatment. Assimilating together the currently available information, a likely scenario for the molecular cascade of noradrenergic differentiation is summarized in Fig. 1.5. The basic pathways between the CNS and PNS are similar except a few points. Generally, exposure of precursor cells to BMPs triggers Mash1 expression, allowing precursors to

become competent for neuronal differentiation. A subset of Mash1 positive cells initiate the expression of Phox2a or Phox2b, which, in turn, directly activate the expression of DBH and TH. Phox2a and Phox2b may also promote morphological features of a neuron by driving the expression of pan-neuronal genes, such as NF160 and peripherin. The close-regulation between Phox2a and Phox2b appears to be essential to maintain each other's expression and to induce DBH and TH expression.

In addition to the three players for noradrenergic specification: BMP, Mash1, and Phox2a/2b, another basic helix-loop-helix transcription factor, dHAND, may modulate the pathway. The expression pattern of dHAND during embryogenesis is confined to developing heart and neural crest derivatives, especially in the autonomic nervous system and adrenal medulla (53). No expression of dHAND is found in the CNS. Inactivation of the dHAND gene did not reveal the direct relevance for the involvement of dHAND in noradrenergic differentiation, because the predominant phenotype of the knock-out mice is embryonic lethal at E10.5 due to heart failure. However, antisense application to block dHAND transcripts in avian neural crest culture results in a significant reduction in generation of catecholamine producing cells (54). Forced expression of dHAND in the developing chick embryo demonstrated more direct evidence to support the role of dHAND in noradrenergic specification; dHAND expression elicits the generation of ectopic sympathetic neurons as detected by Phox2a/2b transcripts (55). Overexpression of either Phox2b or BMP induces dHAND expression in avian embryos. These results position dHAND as a transcriptional modulator downstream of Phox2b in noradrenergic differentiation in the PNS. However, whether dHAND directly activates DBH or TH is

unknown at present. It is still unclear what the natural target genes are and how dHAND activates them.

Overall Goals of This Thesis

In the past five years, a great deal of progress has been achieved in the understanding of Arix/Phox2a and NBPhox/Phox2b, especially in *in vivo* systems, such as loss- and gain-of-function analyses, primary neural crest cultures, and direct avian embryo cultures in collaboration with forced expression of a gene. Yet, the knowledge of the molecular and biochemical aspects of Arix and NBPhox to understand the mechanism of activation of the DBH transcription is still immature.

With respect to homeodomain transcription factors, two major issues need to be addressed. First, in comparison to other transcription factors, the homeodomain proteins are more promiscuous in their DNA binding specificity. In *in vitro*, the same DNA sequence can be recognized by many different homeodomain proteins. Second, natural target genes for most of these proteins are unknown; therefore, little is known of how the homeodomain proteins achieve their functional specificity for the determination of neuronal phenotypes. It is conceivable that target gene specificity may be conferred as a result of posttranslational modification of homeodomain proteins and/or interaction with cofactors that increases the DNA-binding specificity. Thus, biochemical characterization will provide the framework for understanding the functional aspects of homeodomain proteins. The present study takes advantage of the known natural target gene of Arix and NBPhox.

The first part of this thesis focuses on the characterization of Arix and NBPhox with respect to their transcriptional, as well as DNA binding, activities. Furthermore, the transactivation domain of Arix has been investigated. The second part involves insights into the molecular mechanism of Arix in activation of DBH gene transcription in

coordination with activation of the PKA pathway. In particular, I have examined whether the post-translational modification of Arix is functionally relevant to modulate DBH transcription. These studies will provide insight into the molecular mechanism underlying regulation of DBH gene transcription toward noradrenergic cell-type specification.

Table 1.1 Summary of phenotype in Phox2a/2b knock-out mice

	Phox2a -/- mice				Phox2b -/- mice			
	cell morphoogy	expression			cell morphoogy	expression		
		Phox2b	DBH	TH		Phox2a	DBH	TH
locus ceruleus	absent	–	–	ND	E10.5 smaller E18.5 absent	E10.5 + E18.5 –	–	–
sympathetic ganglia	smaller ganglion	ND likely +	+	ND	present but apoptotic	–	–	–
clanial ganglia	present but apoptotic	–	–	ND	present	+	–	ND

ND is not determined.
The table is summarized based on the results from Morin et al. (104) and Pattyn et al. (117).

Figure 1.1. The biosynthetic pathway of catecholamines is illustrated in a presynaptic ending. In addition to catecholamine-synthesizing enzyme, the norepinephrine transporter on a presynaptic plasma membrane is a noradrenergic specific molecule. The receptor for noradrenaline, a, ß-adrenergic receptor, is also depicted on a postsynaptic membrane. A vesicular monoamine transporter on a vesicular membrane appears to be responsible for taking up all catecholamines for storage. Abbreviations: NE=norepinephrine, DA=dopamine, BH_4=tetrahydrobiopterin, DH_2=dihydropterin

Figure 1.1

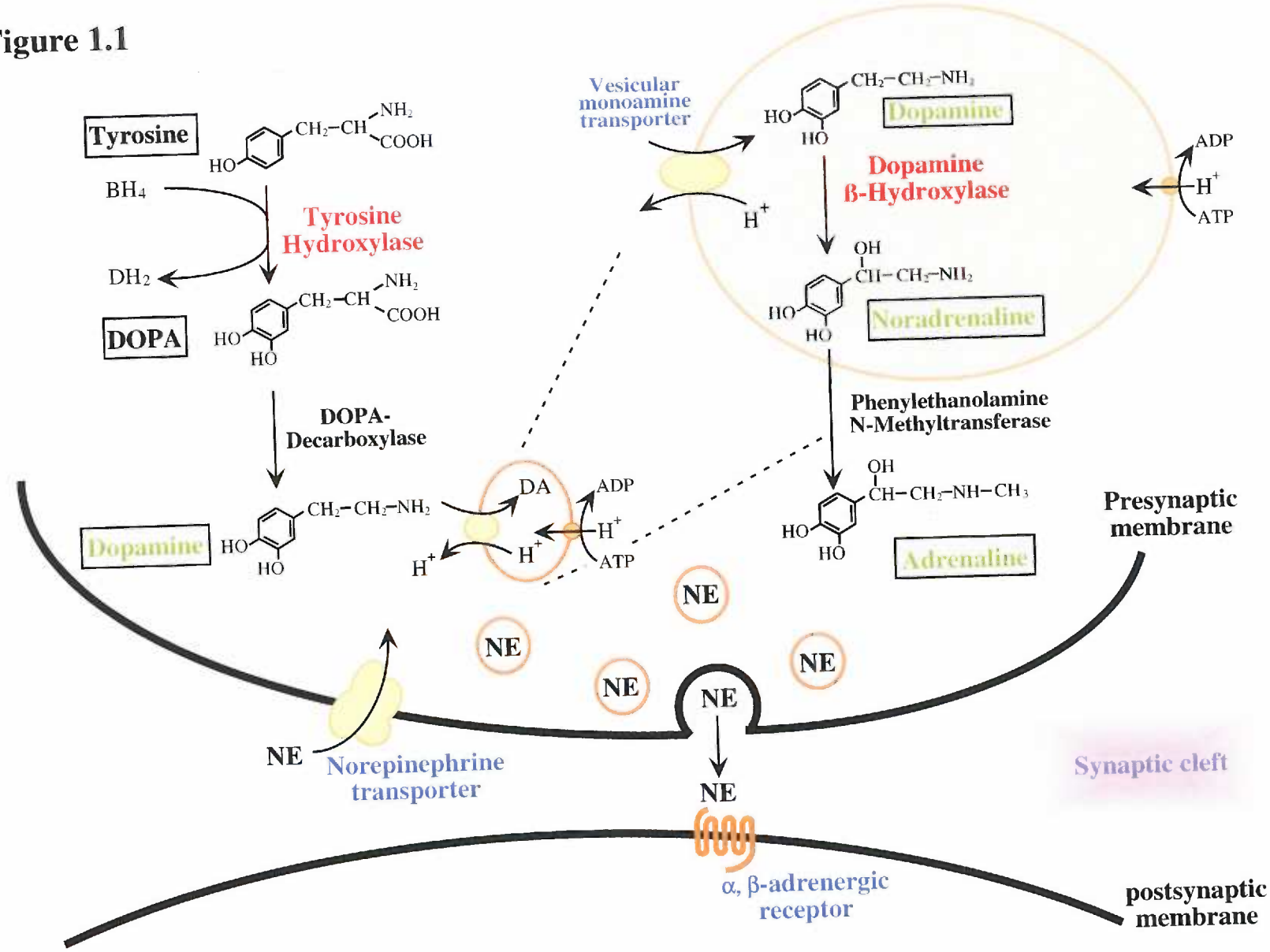

Tyrosine
BH4
Tyrosine Hydroxylase
DH2
DOPA
DOPA-Decarboxylase
Dopamine
Vesicular monoamine transporter
DA
ADP
H+
ATP
Dopamine ß-Hydroxylase
Noradrenaline
Phenylethanolamine N-Methyltransferase
Adrenaline
Presynaptic membrane
NE
Norepinephrine transporter
Synaptic cleft
α, β-adrenergic receptor
postsynaptic membrane

Figure 1.2. A schematic diagram of the DBH promoter is depicted with nucleotide sequences, including –150/–190bp and –56/-73 , which contain a DB1 enhancer and HD3 site, respectively. Abbreviations; CRE=cAMP responsive element, hCREh=human CRE homolog, AP=activator protein, HD=homeodomain recognition site

Figure 1.2

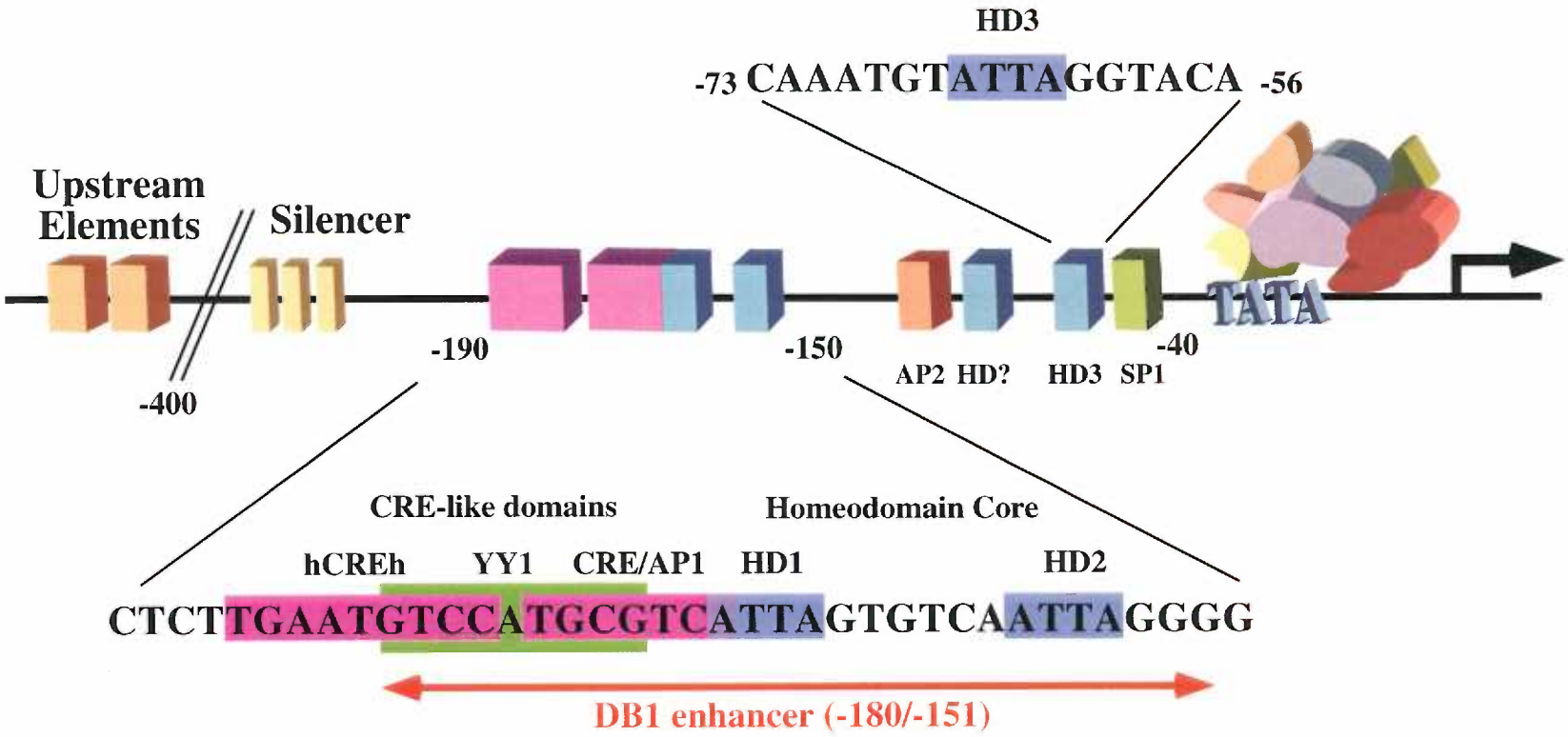

HD3
-73 CAAATGTATTAGGTACA -56
Upstream Elements
Silencer
-400
-190
-150
AP2 HD?
HD3
SP1
-40
TATA
CRE-like domains
Homeodomain Core
hCREh
YY1
CRE/AP1
HD1
HD2
CTCTTGAATGTCCATGCGTCATTAGTGTCAATTAGGGG
DB1 enhancer (-180/-151)

Figure 1.3. A. Arix and NBPhox share high homology, especially in the N-terminus and the homeodomain with 50% and 100% identity, respectively. The homeodomain represents a DNA-binding motif. **B.** The amino acid sequence alignment of Arix and NBPhox is presented. Identical and similar amino acids are boxed and shaded, respectively. Characteristic domains are indicated by colored boxes. **C.** The amino acid sequence within the homeodomain of Arix was compared to the homeodomains of paired and paired-like classes. Percentage of identity is also indicated. A * mark in the helix3 indicates amino acid 50, which categorizes the paired and paired-like homeodomain classes.

Figure 1.3

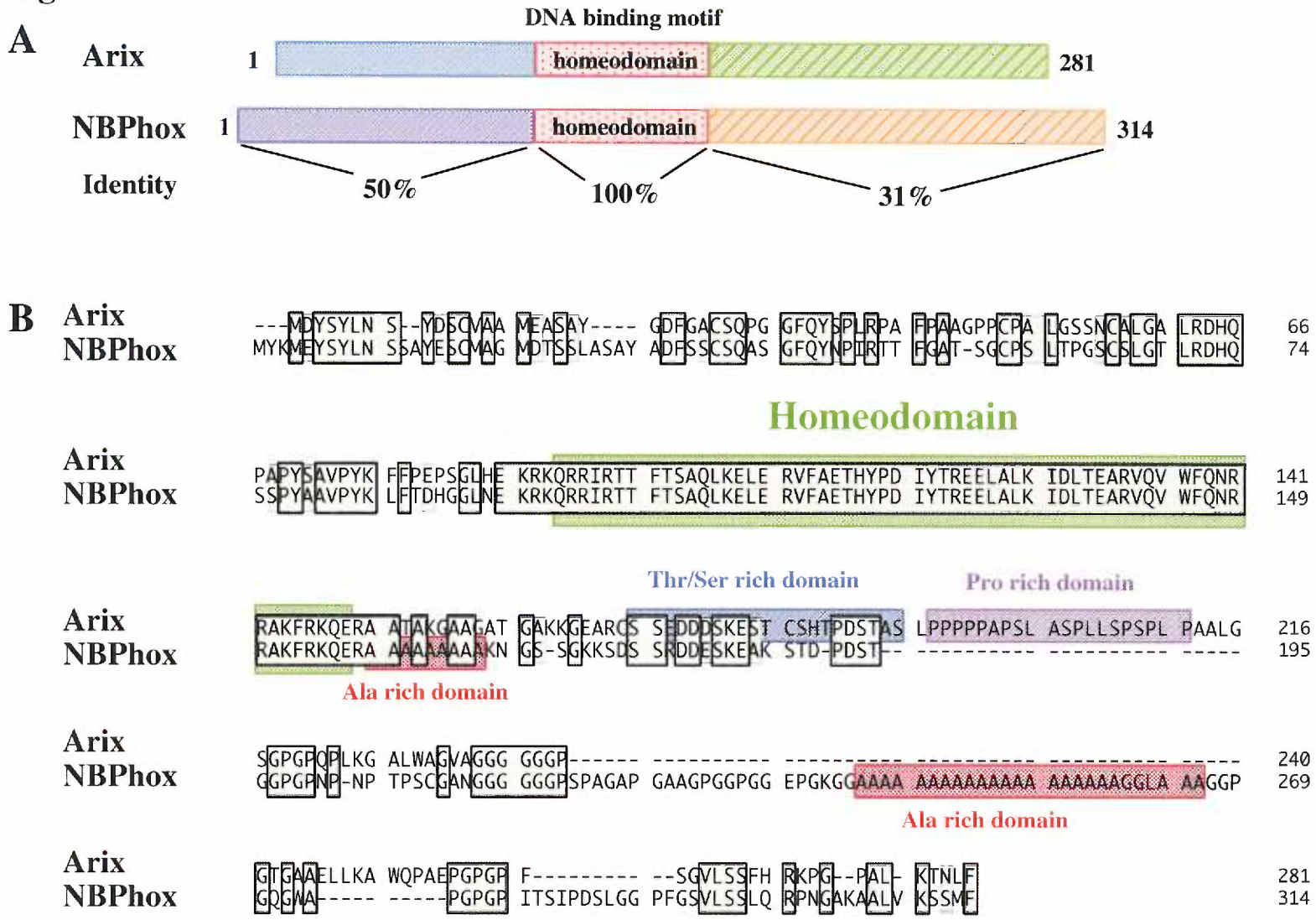

A
DNA binding motif
Arix
1
homeodomain
281
NBPhox
1
homeodomain
314
Identity
50%
100%
31%
B
Arix
NBPhox
Homeodomain
Thr/Ser rich domain
Pro rich domain
Ala rich domain
Ala rich domain

Figure 1.3

C

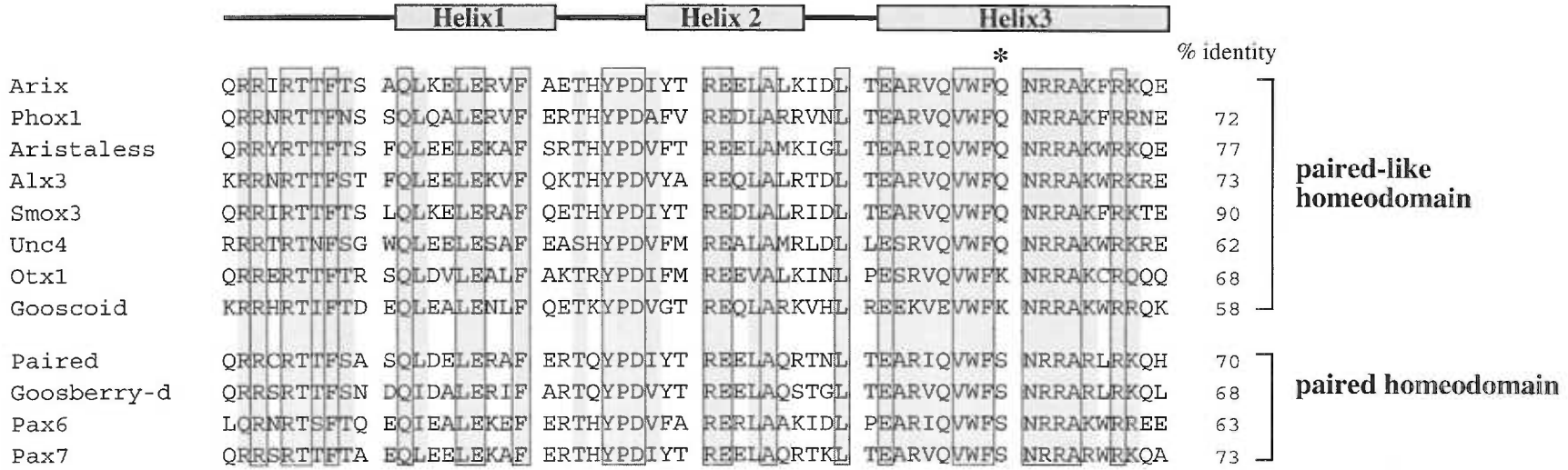
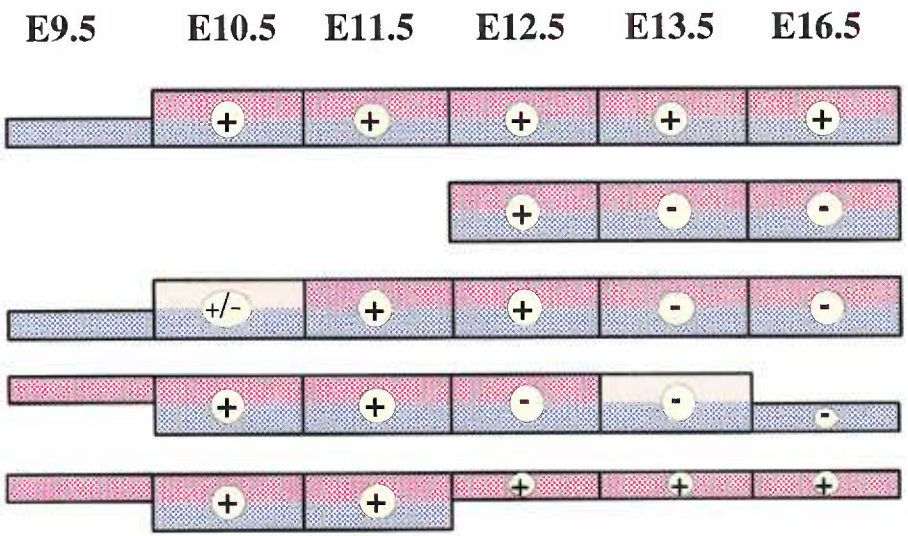

Helix1
Helix 2
Helix3
*
% identity
Arix QRRIRTTFTS AQLKELERVF AETHYPDIYT REELALKIDL TEARVQVWFQ NRRAKFRKQE
Phox1 QRRNRTTFNS SQLQALERVF ERTHYPDAFV REDLARRVNL TEARVQVWFQ NRRAKFRRNE 72
Aristaless QRRYRTTFTS FQLEELEKAF SRTHYPDVFT REELAMKIGL TEARIQVWFQ NRRAKWRKQE 77
Alx3 KRRNRTTFST FQLEELEKVF QKTHYPDVYA REQLALRTDL TEARVQVWFQ NRRAKWRKRE 73
Smox3 QRRIRTTFTS LQLKELERAF QETHYPDIYT REDLALRIDL TEARVQVWFQ NRRAKFRKTE 90
Unc4 RRRTRTNFSG WQLEELESAF EASHYPDVFM REALAMRLDL LESRVQVWFQ NRRAKWRKRE 62
Otx1 QRRERTTFTR SQLDVLEALF AKTRYPDIFM REEVALKINL PESRVQVWFK NRRAKCRQQQ 68
Gooscoid KRRHRTIFTD EQLEALENLF QETKYPDVGT REQLARKVHL REEKVEVWFK NRRAKWRRQK 58
Paired QRRCRTTFSA SQLDELERAF ERTQYPDIYT REELAQRTNL TEARIQVWFS NRRARLRKQH 70
Goosberry-d QRRSRTTFSN DQIDALERIF ARTQYPDVYT REELAQSTGL TEARVQVWFS NRRARLRKQL 68
Pax6 LQRNRTSFTQ EQIEALEKEF ERTHYPDVFA RERLAAKIDL PEARIQVWFS NRRAKWRREE 63
Pax7 QRRSRTTFTA EQLEELEKAF ERTHYPDIYT REELAQRTKL TEARVQVWFS NRRARWRKQA 73
paired-like homeodomain
paired homeodomain

Figure 1.4. The relative timing of Arix/Phox2a, NBPhox/Phox2b, and DBH expression during development is summarized in major noradrenergic tissues. Lighter and darker shading indicate the weak and strong expression, respectively. +/- signs in yellow circles represent the level of DBH expression. The presentation is modified from Tiveron et al.(156), and Pattyn et al. (116).

Figure 1.4



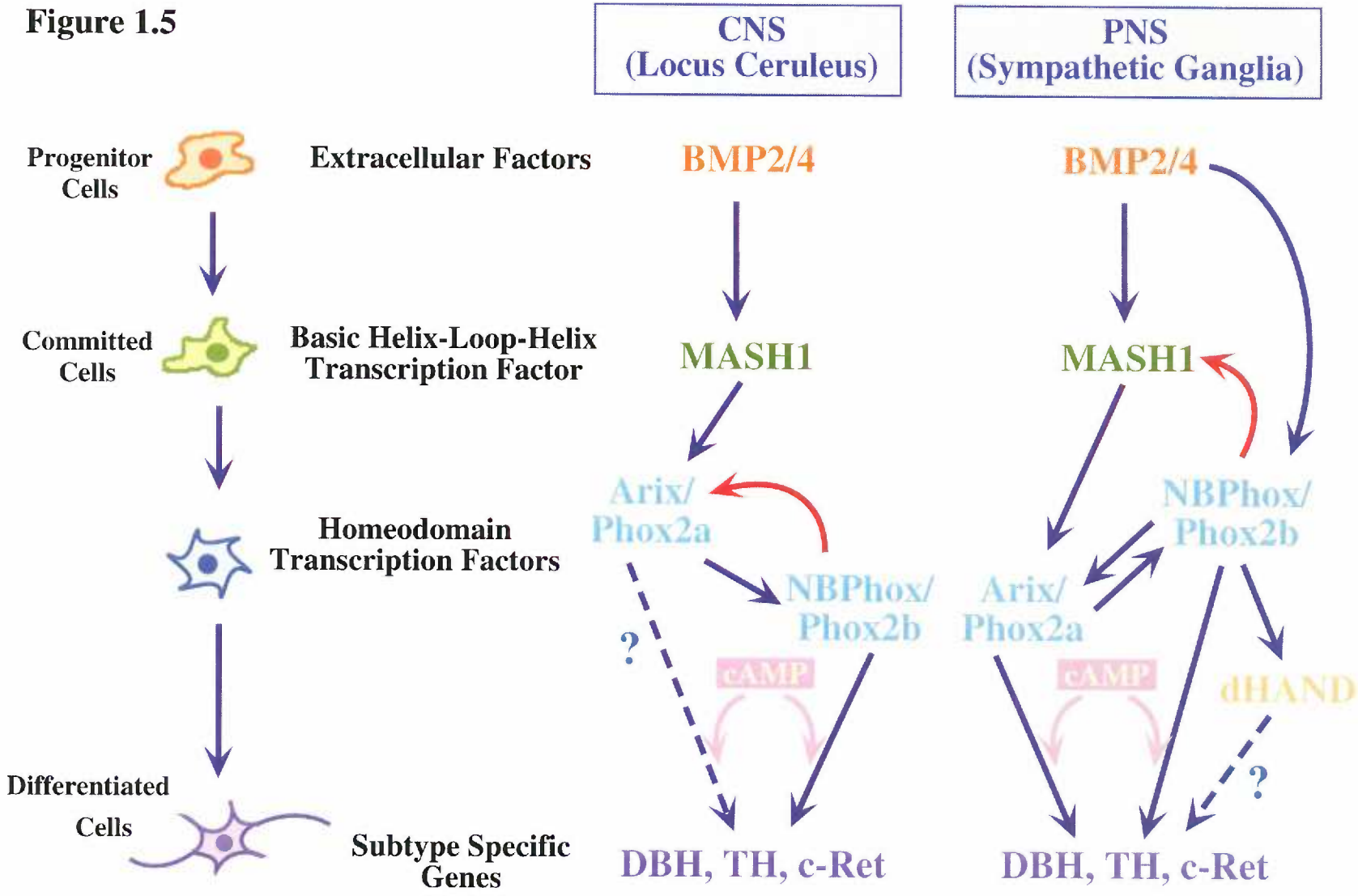
Arix/Phox2a expression

NBPhox/Phox2b expression

⊕ DBH expression

Figure 1.5. based on the current findings, the pathways for noradrenergic neuronal differentiation in the CNS and PNS are illustrated. Blue arrows indicate direct induction of a gene. Red arrows indicate requirement to maintain expression of a gene. Dashed arrows imply that induction of a gene is not known whether it is direct or indirect.

Figure 1.5


CNS
(Locus Ceruleus)
PNS
(Sympathetic Ganglia)
Progenitor Cells
Extracellular Factors
BMP2/4
BMP2/4
Committed Cells
Basic Helix-Loop-Helix Transcription Factor
MASH1
MASH1
Arix/
Phox2a
NBPhox/
Phox2b
Homeodomain Transcription Factors
NBPhox/
Phox2b
Arix/
Phox2a
?
cAMP
cAMP
dHAND
?
Differentiated Cells
Subtype Specific Genes
DBH, TH, c-Ret
DBH, TH, c-Ret

Chapter Two

Paired-like Homeodomain Proteins Phox2a/Arix and Phox2b/NBPhox Share Similar Genetic Organization and Independently Regulate Dopamine β-Hydroxylase Gene Transcription

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RUNNING TITLE
Genetic and biochemical studies of Phox2a/Arix and Phox2b/NBPhox.

ABSTRACT

The homeodomain transcription factors Arix/Phox2a and NBPhox/Phox2b play a role in the specification of the noradrenergic phenotype of central and peripheral neurons. To better understand the functions of these two factors, we have compared the genetic organization, chromosomal location and transcriptional regulatory properties of Arix and NBPhox. The gene structure is very similar, with each gene containing three exons and two introns, extending a total of approximately 5 kb. Arix and NBPhox are unlinked in human and mouse genomes. NBPhox is located on human Chromosome 4p12 and mouse Chromosome 5, while Arix is located on human Chromosome 11q13 and mouse Chromosome 7. Both proteins bind to three sites in the promoter proximal region of the rat dopamine β-hydroxylase gene. Arix and NBPhox form DNA-independent multimers *in vitro*, and exhibit cooperative binding to the DB1 regulatory element, which contains two homeodomain recognition sites. Both proteins regulate transcription from the rat dopamine β-hydroxylase promoter, and transcription is synergistically increased in the presence of PKA plus either Arix or NBPhox. The two transcription factors exhibit similar concentration dependent efficacies, and when coexpressed, transcription is stimulated to a value approximately equal to either factor alone. The N-terminal segment of Arix is essential for transcriptional regulatory activity, and this region bears 50% identity with NBPhox, suggesting a similar mechanism in transcriptional activation of the DBH gene. We conclude from this study that Arix and NBPhox exhibit indistinguishable and independent transcriptional regulatory properties on the DBH promoter.

INTRODUCTION

The identity and functional properties of a neuroendocrine cell are dependent upon the expression of the appropriate complement of gene products within the cell. For a neuronal cell, certain gene products, such as neurofilament proteins and peripherin, are common to most neurons, and are termed pan neuronal genes. Other proteins are specific to a particular neuronal phenotype and are referred to subtype specific gene products. Proteins in the latter class include neurotransmitter receptors, ion channels and neurotransmitter biosynthetic enzymes. In order for appropriate function of a neuroendocrine cell type to occur, it is essential that the correct array of gene products is selectively maintained.

Determination of the noradrenergic phenotype of a neuron during development appears to be under the control of two similar homeodomain transcription factors, Phox2a/Arix and Phox2b/NBPhox (104, 117). Phox2a/Arix and Phox2b/NBPhox are members of the paired-like family of homeodomain transcription factors, and share significant sequence homologies, including 100% identity within the homeodomain and 50% identity in the N-terminal region to the homeodomain (116, 159, 171-173). The expression patterns of Phox2a/Arix and Phox2b/NBPhox are spatially and temporally overlapping but distinct in both the central and the peripheral nervous systems during embryogenesis (116, 156). Expression is primarily restricted to cells which transiently or permanently transcribe genes responsible for noradrenaline biosynthesis, including

tyrosine hydroxylase (TH)[2] which is the rate limiting enzyme of the catecholamine biosynthetic pathway, and dopamine β-hydroxylase (DBH), the enzyme which catalyzes the conversion of dopamine to noradrenaline. Mice containing a null mutation of the Phox2a gene exhibit complete loss of the central noradrenergic cells of the locus ceruleus, and cease the transient expression of DBH in migrating peripheral sensory and autonomic neuroblasts (104). Yet, some neuronal populations that normally express Phox2a/Arix, including those in the sympathetic chain ganglia, continue to synthesize DBH in the *Phox2a-/-* mice. The ganglia in these mice express Phox2b, leading to the suggestion that Phox2b would substitute for Phox2a in the regulation of genes necessary for noradrenaline biosynthesis. Mice containing a targeted deletion of Phox2b, in fact, fail to develop mature sympathetic and parasympathetic ganglia, lose appropriate expression of Phox2a and DBH, and die before birth (116). Further support of the role of these two proteins in noradrenergic phenotype determination is evidence that a dominant negative construct of Phox2a/Arix will inhibit TH and DBH expression in rat neural crest cell cultures (88) and that overexpression of Phox2a or Phox2b in the chick embryo promotes the generation of ectopically located neurons expressing DBH and TH (145). These targeted mutations and overexpression experiments suggest that Phox2a/Arix and Phox2b/NBPhox may have redundant or interactive roles in activating the target genes associated with the noradrenergic phenotype.

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[2] The abbreviations used are: TH, tyrosine hydroxylase; DBH, dopamine β-hydroxylase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; HA, hemaglutinin; HD, homeodomain; IVT, *in vitro* translated; HST, Herstatin; EGFP, enhanced green fluorescent protein; HRP, horse radish peroxidase

Phox2a/Arix and Phox2b/NBPhox are believed to regulate the production of noradrenaline through direct interaction with regulatory elements on the 5' flanking segments of the TH and DBH genes. Defined homeodomain binding sites have been identified and characterized in the DBH gene, and elimination of these sites influences basal activity from the DBH promoter (69, 150, 170, 173). In cell transfection assays, Phox2a/Arix and Phox2b/NBPhox exert modest stimulatory activity from the DBH and TH promoters (150, 170, 173). In addition, Phox2a/Arix synergizes with the cAMP pathway to produce strong activation of the DBH (150), and TH (88) genes. These results suggest a link between Phox2a/Arix and extracellular stimuli, which together influence the specificity of gene expression.

The homeodomains of several paired-type homeodomain transcription factors have been shown to exhibit cooperative interactions between two closely spaced homeodomain binding sites. The cooperative interactions may result from homo- or heterodimerization with other transcription factors in the same class (121, 157, 165). However, for most of these proteins, true target genes are unknown, and so the interaction of these proteins with closely spaced homeodomain binding sites in authentic regulatory sites, within the context of a target gene promoter, has not been addressed. The DBH gene contains two homeodomain regulatory sites separated by 6 bases. This naturally occurring regulatory site provides an ideal system to evaluate potential cooperativity of binding of Phox2a/Arix and Phox2b/NBPhox to the DBH gene, as well as their interaction with each other.

The identification of these two proteins with identical homeodomains suggests possible functional redundancy in regulating the transcriptional specificity of the DBH

gene. Since the homeodomain contains the DNA binding motif, it may be anticipated that Phox2a/Arix and Phox2b/NBPhox would share target specificity. The two proteins could function independently, cooperatively, or synergistically to increase transcription of target genes. Alternatively, Phox2a/Arix and Phox2b/ NBPhox could antagonize each other's activity.

In the present study, we have characterized genetic, functional, and biochemical aspects of NBPhox and have compared its properties with Arix. We have determined the NBPhox gene structure and have mapped the gene in both human and mouse chromosomes. Our findings demonstrate that the organization of the Arix and NBPhox genes are similar to each other but are located on different chromosomes. We also show that Arix and NBPhox similarly and independently function in activating DBH transcription, and bind to the multiple target sites within the DBH promoter with moderate cooperativity. *In vitro* protein-protein interaction assays indicate physical interactions between Arix and NBPhox, which may contribute to the cooperative binding to the homeodomain binding sites in the DBH promoter. In addition, we demonstrate that the N-terminal segment (aa1-83) of Arix, bearing high homology to NBPhox, is necessary for the transcriptional activity of Arix, suggesting that Arix and NBPhox may share a similar mechanism to regulate the DBH gene expression.

MATERIALS AND METHODS

Cell culture— HepG2 cells were cultured in minimum Eagle's medium (MEM) supplemented with 10% fetal bovine serum (Hyclone), 1% non-essential amino acids, and 110 mg/l sodium pyruvate. NT2 cells were cultured in Dulbecco's modified Eagle's medium with an addition of 10 % fetal bovine serum. Both cell lines were maintained at 37 °C in an atmosphere of humidified air containing 5 % CO_2.

Amplification of DNA by the polymerase chain reaction (PCR)— For amplification of Pmx2b DNA in mouse genomic DNA, the primer pair 5'-GCACTACCCTGACATCTACACC-3' (corresponding to bases 578-590 of mouse Phox2b) and 5'TCCTGCTTGCGAAACTTAGC-3' (corresponding to bases 668-687 of mouse Phox2b) was used. PCR reactions contained 62.5 ng of template DNA, 10mM Tris-HCl (pH9.0 at 25 °C), 50mM KCl, 0.1% Triton X-100, 1.5mM $MgCl_2$, 50 ng primers, and 200nM deoxynucleotide triphosphates in a total volume of 25 µl. Reactions were heated for 5 min at 94 °C, 2.5u Taq polymerase (Promega) was added, and reactions were cycled at 94 °C for 1 min, 57 °C for 1 min and 72 °C for 2 min, for a total of 30 cycles. The resulting DNA fragments were resolved by electrophoresis with 2% agarose. For mapping of human NBPHOX DNA, two regions of intronic NBPHOX DNA were amplified. A 400 base segment between introns 1 and 2 was amplified with primer pairs 5'-CGGCTGATT TGCTCACTTTCTG-3' and 5'-CTAAGCCTCTCTCGAACGCACAG-3', and a 312 base segment in intron 2 was amplified with primer pairs 5'-CACGCTCTTCC

AGGCTCAAAG-3' and 5'-CGCGCACATC CACCAAGTC-3'. PCR reactions contained the same reagents as described above for mapping of mouse *Pmx2b*. For the PCR reaction, samples were heated at 94 °C for 5 min, and then cycled 30 times at 94 °C for 1 min, 64 °C for 1 min and 72 °C for 1min. The mapping results were consistent between the amplified products derived from the two primer pairs.

Cloning of human NBPhox cDNA— The cDNA for human NBPhox was constructed by a combination of polymerase chain reaction (PCR) and genomic cloning techniques. DNA encoding the N-terminal and homeodomain portion of NBPhox were cloned by reverse transcriptase-PCR of RNA from SHSY-5Y neuroblastoma cells, using primers 5'-TGCTCTAGAGACCTCAGACAAG G-3' and 5'-GAAGAGTCAGACTTTTTGCCCG-3', encompassing bases 174 to 896 of the original NBPhox cDNA (Yokoyama *et al.*, 1996). The C-terminal and 3' untranslated segments are encoded within one exon, and were identified by isolation of a genomic clone of human NBPhox. The two segments were recombined by cloning techniques, generating an NBPhox cDNA corresponding to bases 177 to 1831 of the original cDNA. This cDNA contains the entire coding sequence, 185 bases of 5' and 533 bases of 3' untranslated regions.

Plasmid constructs— The construction of plasmid containing the promoter and 5'-flanking sequence of DBH gene cloned adjacent to the bacterial chloramphenicol acetyltransferase (CAT) transcription unit was described previously (139). The DBH-Luc (-232/+10) reporter plasmid was constructed by insertion of a *Hind*III-*Mlu*I

fragment, the digested PCR product derived from DBH-CAT(-232/+14) (139), into the luciferase reporter vector, pGL3 (Promega). This reporter construct contains the proximal DBH promoter (-232/+10) including the DB1 enhancer region, TATA-like sequence, and the transcription start site, linked upstream of the coding sequence of firefly (*Photinus pyralis*) luciferase.

A hemaglutinin (HA) epitope tagged rat Arix expression plasmid (HA-Arix) was constructed using vector HA6.1, which consists of pcDNA3 (Invitrogen) with an HA epitope tag. HA6.1 was generously provided by Dr. Paul Shapiro. To construct HA-Arix, the DNA encoding the N-terminal and homeodomain regions of Arix were amplified by PCR. The 5' primer, 5'-GGGGTTCTTGAATTCCATATGGACTACTCCTAC CTCAT-3' encodes the first 5 amino acid residues of N-terminal Arix and the 3' primer, 5'-GGCTGCACG TGGACTCCTTGGA-3' represents the complement sequence of residues 725-746, distal to the homeobox. The amplified segment was fused to the C-terminal portion of Arix and cloned into HA6.1 as an EcoRI/NotI fragment. The resultant plasmid contains the complete rat Arix coding sequence fused in frame with HA tag on the N-terminus and 313 bases of 3' untranslated sequence. To construct HA-ArΔC, full length Arix was digested with BssHII and ScaI. The BssHII site was filled in with Klenow and ligated to the blunt ScaI end. To construct HA-ArΔN, the Arix cDNA was amplified with the primer 5´-CCAGGTA CCATGGT GCTGCATGAGA AGCGCAAGCAG-3', containing a methionine codon adjacent to codons for the 6 amino acids preceding the homeodomain, and a primer distal to the homeodomain described above. The PCR product was subsequently fused to the C terminal segment of the Arix cDNA and cloned

into HA6.1. The ArHD construct, encoding only homeodomain, was made from ArΔN by further truncation of the C-terminus as described in HA-ArHD construct. After removal of the C-terminus of Arix, ArΔC was subcloned into HA6.1. To construct the NBPhox mammalian expression plasmid, CMV-NBPhox, cDNA containing the full coding sequence of NBPhox plus 184 and 525 bases of 5' and 3' untranslated sequences respectively was subcloned into pcDNA3 (InVitrogen). RSV-PKA expression vector was a generous gift from Dr. Richard Maurer (96).

Transfections— DNA used for transfections was purified by equilibrium centrifugation in $CsCl_2$-ethidium bromide gradient. For HepG2 cell transfections, cells were plated at a density of 3×10^6 in a 100 mm dish one day before transfection and transfected with same amounts of DNA (8.5 μg) by calcium phosphate precipitation as described previously (41, 139). For NT2 cell transfections, 0.5×10^6 NT2 cells per well in a 6 well plate were transfected with same amounts of DNA (1.85 μg) using PerFect Lipid-8 (Invitrogen), according to the manufacturer's instructions. During transfection, NT2 cells were incubated in OPTIMEM 1 (GIBCO/BRL) with a lipid-DNA mixture at a ratio of 1.5:1 for 4 hr. The total amounts of DNA were adjusted using HA6.1, the backbone expression vector. Cells were harvested 24 or 48 hr after transfection and aliquots of cell extracts were assayed for protein content, CAT activity (40), and/or luciferase activity using Dual-Luciferase Assay System (Promega). All transfections contained a luciferase plasmid, either from firefly or jellyfish (Renilla), under control of the CMV promoter and enhancer, for use as an internal control for transfection efficiency. However, we found that PKA stimulated the CMV promoter and enhancer, and thereby would distort the

experimental results if luciferase values were used to standardize transfection efficiencies. Therefore, values presented for reporter gene activity are standardized to protein concentration per extract. The values of standardized data using protein concentration differ from those using a luciferase standard quantitatively, but the qualitative relationships between experimental conditions are the same.

Expression of recombinant Arix and NBPhox— To produce recombinant rat Arix and human NBPhox, cDNAs were subcloned into the prokaryotic expression vector pET30 (Novagen), which carries DNA encoding an N-terminal 6xHis tag. pET30 plasmids containing His-NBPhox or His-Arix cDNAs were transformed into bacterial host BL21(DE3). To induce production of recombinant proteins, bacteria in log phase were treated with 1mM IPTG, followed by incubation at 37 °C for 4 hr. Bacteria were pelleted, and resuspended in sonication buffer (50mM Tris-HCl pH8, 500mM NaCl, 1% CHAPS, 0.2mM phenylmethylsulfonylfluoride, and 10mM sodium fluoride). Bacterial suspensions were sonicated to lyse bacteria. Soluble fractions were collected after centrifugation and applied to TALON metal affinity resin (Clontech) for purification of recombinant Arix. For NBPhox, bacterial pellets were further solubilized in 50mM CAPS (pH11) supplemented with 3% N-laurylsarcosine. The resultant solubilized fraction was dialyzed against the renaturing buffer (20mM Tris-Cl pH8, 100 mM NaCl, and 0.1mM dithiothreitol). After the removal of dithiothreitol by dialysis, the fraction containing soluble NBPhox was applied to the Ni resin (Qiagen). His-tagged recombinant proteins were eluted by step gradients of imidazole containing elution buffer. The protein concentration of His-Arix was determined by Bradford assay (Bio

Rad) and the concentration of His-NBPhox was estimated by western blots using Ni-labeled HRP (Kirkegaard&Perry Lab) in comparison to His-Arix. For convenience, the concentration of recombinant protein was expressed in arbitrary units and each unit approximately contains the same amount of His-Arix or His-NBPhox.

Electrophoretic Mobility Shift Assays (EMSA)—Synthetic sense and antisense oligonucleotides were end-labeled with P^{32}-γATP using T4 polynucleotide kinase and then annealed. EMSA were carried out in 20 μl of the binding reaction buffer (12.5mM Hepes pH7.9, 5mM $MgCl_2$, 50mM KCl, 1mM EDTA, 1mM dithiothreitol, 2μg of poly(dI-dC)·poly(dI-dC), and 10% glycerol). Labeled probe (20,000cpm Cerenkov counts) and recombinant Arix and/or NBPhox were added in the binding reaction followed by 30min incubation on ice. For the competition assay, the binding reactions were preincubated for 20min in the presence of unlabeled double-stranded oligonucleotides prior to the addition of labeled oligonucleotides. DNA-protein complexes were resolved on 6% non-denaturing polyacrylamide gels (19:1 = acrylamide: bisacrylamide) in electrophoresis buffer (45mM Tris borate and 1mM EDTA) followed by autoradiograph. For the quantitation of EMSA, relative proportions of bound probes were calculated using a phosphoimager. The data points best fit to the Hill equation, $A = A_{max}X^h/(K_x{}^h+X^h) + A_0$, where A is fraction of bound, X is protein concentration, A_{max} is fractional saturation, A_0 is initial fraction, K_x is half fractional concentration, and h is the Hill coefficient index.

Cooperativity between HD1 and HD2 sites within the DB1 was measured based on the cooperative factor, τ, described by Wilson *et al.* (165). τ is defined as K_{d2}/K_{d1},

where K_{d1} and K_{d2} are the dissociation constants for the following reactions.

$$P + D \xrightarrow{K_{d1}} PD \qquad K_{d1}=[PD]/2[P][D]$$

$$PD + P \xrightarrow{K_{d2}} P_2D \qquad K_{d2}=2[P_2D]/[PD][P]$$

P is the protein, D is the unbound DNA, PD is the singly occupied DNA, and P_2D is the doubly occupied DNA. Therefore,

$$\tau = K_{d2}/K_{d1} = 4[P_2D][D]/[PD]^2$$

The cooperativity factor, τ, describes the strength of cooperativity in which one site occupancy aids the second site occupancy. τ is calculated at 50% of free probe.

Immunocytochemistry—HepG2 cells were plated at a density of $1x10^5$ cells in a 4-well chamber slide one day before transfection. Cells were transfected with 100 ng of pEGFP-C1 (Clontech) and 100ng of a given truncated or full-length Arix construct using Efectene (Qiagen) according to the manufacturer's instruction. 24hr after transfection, cells were fixed with 4% paraformaldehyde and transfected cells were visualized by enhanced green fluorescent protein (EGFP). HA-tagged full-length and truncated Arix, were detected by immunofluorescence or HRP (horseradish peroxidase) reactivity. For immunofluorescence detection, fixed cells were stained with rat anti-HA antibody (Roche) followed by biotinylated rat IgG (Vector) and incubated with rhodamine avidinD (Vector labs). For HRP-based detection, fixed cells were stained with anti-HA antibody followed by incubation with biotinylated secondary antibody and ABC complex (Vector

labs). The resultant immuncomplexes were developed with diaminobenzidine reaction products.

***In vitro* translation and *in vitro* protein-protein interaction assay**— Arix, NBPhox, cfos, and Herstatin constructs were transcribed by T7 RNA polymerase and translated in the presence of ^{35}S-methionine using TNT Coupled Wheat Germ Extract System (Promega) according to the manufacturer's instructions. Arix was cloned in HA6.1 vector, and NBPhox, rat c-fos, and Herstatin (26) were cloned in pcDNA3.

In the *in vitro* protein-protein interaction assay, 5 μg of soluble fractions containing bacterially expressed His-Arix or His-NBPhox fusion proteins were conjugated to TALON metal affinity resin (Clontech) and co-incubated with 3 μl of *in vitro* translated ^{35}S-labeled proteins in 250 μl of the binding buffer (10mM Tris-HCl, 200mM NaCl, 20mM imidazole, and 1mg/ml BSA adjusted to pH8) for 2 hr at 4 °C. After the incubation, resin was washed three times with 1 ml of the binding buffer containing 0.25% NP-40, followed by two additional washes with 1 ml of the binding buffer without NP-40. The resin was boiled in SDS-PAGE sample buffer for 5 min and subjected to SDS-PAGE, followed by autoradiography. In some experiments, the resin was further treated with micrococcal nuclease to eliminate DNA contaminants (78). The resin-protein conjugates were subsequently washed and resuspended in 50 μl of digestion buffer (10mM Tris pH8, 50mM NaCl, and 4mM $CaCl_2$) after the protein-protein interaction assay described above. The resin was incubated with 0.4 unit of micrococcal nuclease at 37 °C for 1hr and washed with 1 ml of the digestion buffer and 1 ml of the binding buffer without NP-40 prior to SDS-PAGE.

RESULTS

Genomic structures of human Arix and NBPhox

To compare the gene organization of human Arix and NBPhox, genomic clones of each were isolated from a human genomic library. Subclones were sequenced to define intron and exon regions. The nucleotide sequences of Arix and NBPhox can be accessed through GenBank[3]. Several similarities in the exon/intron structures of these two genes are apparent (Fig 2.1A). The size of the transcribed portions of both genes is similar, at approximately 5 kb. Both genes are divided into three exons, with exon 3 the longest. Amino acids in exon 1 share 45% identity between human Arix and NBPhox (Fig 1B). Exon 2 is largely comprised of the homeodomain, identical between Arix and NBPhox, while exon 3 residues exhibit little homology, other than those that are part of the homeodomain.

In both genes, the homeodomain is split between exons 2 and 3, and the position of the split is identical, between amino acids Q46 and V47 of the third helix of the homeodomain, which makes a direct contact with the recognition sequence (ATTA/TAAT as a core) of DNA (Fig 2.1B) [see reference (35)]. This position at which the DNA encoding the homeodomain is split between exons 2 and 3 is also found in other paired-type homeodomain genes, including the Otx (142) and goosecoid (11, 34) families and Phox1[4]. In addition, these genes are organized into 3 exons, similar to the structure found with Arix and NBPhox. The similarities between these genes suggest a common

[3] GenBank accession numbers for Arix and NBPhox are AF117979, AF02272, AF02273, and AF02274.
[4] GenBank accession numbers for Phox1 is Z97200.

origin for these paired-like homeodomain protein families.

Mapping of chromosomal location of the mouse homolog of NBPhox, *Pmx2b*

To determine the chromosomal location of the mouse homolog of Phox2b/ NBPhox, *Pmx2b*,[5] DNA mapping panels derived from matings of the mouse strains C57BL/6J and SPRET/Ei (Mus spretus, Spain) were typed for *Pmx2b*. A PCR fragment of *Pmx2b* which differs in size between the two parental strains was used to define the parental origin of *Pmx2b* alleles in the DNAs of the mapping panel. DNA was amplified between exons 2 and 3 resulting in a fragment of approximately 1.1 kb. Using electrophoresis with 2% agarose, a small difference in size can be resolved between the C57BL/6J and SPRET parents.

The two DNA panels used to map NBPhox were obtained from The Jackson Laboratory Backcross DNA Mapping Panel Resource. One panel consists of DNA from 94 individual mice from a backcross of (C57BL/6J x M spretus) x C57BL/6J (panel BSB), while the second panel consists of DNA from 94 individual mice from the reciprocal backcross (C57BL/6JEi x SPRET/Ei) x SPRET/Ei (panel BSS). The BSS panel has been typed for over 4000 different polymorphic loci, while the BSB panel has been typed for over 900 different loci (128).

Genetic linkage was analyzed by comparing the segregation pattern of NBPhox alleles among the backcross progeny with the segregation patterns of previously mapped loci. The computer program Map Manager was used to perform linkage and haplotype

[5] The locus for mouse NBPhox (*Phox2b*), has already been named Pmx2b (Mouse Genome database). For consistency, we have named the human locus PMX2B, and have registered this name with the HUGO Nomenclature Committee.

analysis. Gene order on a chromosome was determined by minimizing the number of double crossover events required to explain the observed haplotype distributions. The data from the linkage analysis is assigned the Mouse Genome Database (MGD) accession number J:45820 and can be accessed at URL: www.informatic.jax.org.

In both backcrosses, significant linkage was found with loci in the middle of Chromosome 5. *Pmx2b* maps one crossover distal to marker *D5Mit15* in the BSB cross and co-segregates with this same marker in the BSS cross (Fig 2.2A). The combined data from the two crosses gives the following order: proximal-*D5Mit15* - 0.53 cM ± 0.53 cM - *Pmx2b* - 3.19cM ± 1.28cM -*D5Nds2* -distal. On the basis of these results, our best estimate of the position for the gene locus Pmx2b is at offset 39 cM on mouse Chromosome 5 (Fig 2.2B), in a region that shares extensive conserved linkage with human Chromosome 4. *Pmx2b* is not linked to *Arix*, which maps to distal Chromosome7 (63).

Mapping of chromosomal location of human NBPhox

To map the chromosomal location of the human gene encoding NBPhox, designated PMX2B, DNA mapping panels from somatic cell hybrids between rodent and human genomes were used. The first panel used was NIGMS mapping panel, where each DNA sample contains a defined complement of human chromosomes, as well as the complete genome of the rodent cell line from which the hybrid was derived. Concordance analysis from these results indicated that PMX2B is located on human Chromosome 4.

To confirm and further refine the localization of PMX2B, a second DNA mapping

panel, the GeneBridge 4 radiation hybrid panel, was used. This panel contains DNA samples from 93 radiation hybrid clones of the whole human genome. It was developed from exposure of a human cell line with X-ray followed by fusion with hamster recipient cells. DNA samples were analyzed for the presence of PMX2B, and the pattern was compared relative to the Whitehead Institute/MIT Center for Genome Research's radiation hybrid map. This analysis placed PMX2B on Chromosome 4p12, at 2.84 cR from marker D4S496 (lod>3) (Fig 2.2C). This is approximately 9cR distal to ubiquitin carboxyl-terminal hydroxylase (UCH-L1) and 35cR from the location of the genes encoding the GABA receptor subunit B1, GABRB1-A and GABRB1-B. PMX2B is not linked to ARIX, which maps to human Chromosome 11q13 (63).

Characterization of Arix and NBPhox in the binding to the DBH promoter

Within the DBH promoter (-232/+14) are three homeodomain (HD) protein binding sites, containing ATTA as a core recognition sequence (Fig 2.3A). Two of the sites, HD1 and HD2, are separated by 6 bases and located adjacent to the CRE/AP1 site in the DB1 enhancer, which is involved in noradrenergic tissue-specific regulation, Arix-dependent activation, and PKA-induced modulation (Shaskus *et al.*, 1992; Zellmer *et al.*, 1995; Swanson *et al.*, 1997, 1998; Kim *et al.*, 1998;). The third homeodomain binding site, HD3, is positioned at -67 and is flanked by bases (TGATTAG), similar to those of HD1 (TCATTAG). The HD1/2 sites are necessary for a maximal response to PKA-mediated DBH promoter activation, while the HD3 site enables general promoter activation (Swanson *et al.*, 1997, 2000; Kim *et al.*, 1998). To compare the functional

roles of Arix and NBPhox, we have characterized DNA binding activities of these proteins with the homeodomain recognition sites in the DBH promoter.

To characterize the binding of Arix and NBPhox to the DBH promoter, full-length recombinant His-Arix and His-NBPhox were bacterially produced and electrophoretic mobility shift assays (EMSA) were carried out using oligonucleotides bearing the HD3 site or both the HD1 and HD2 sites, as well as oligonucleotides with mutations at these HD sites (Fig 2.3B). Both His-Arix and His-NBPhox form a DNA-protein complex with the ATTA probe, containing the HD3 site (Fig 2.3C). This DNA-protein complex is abolished by the addition of an unlabeled ATTA oligonucleotide, and failed to form when an oligonucleotide (HD3m) containing a mutation in the homeodomain binding site is used as a probe, confirming the specificity of His-Arix and His-NBPhox binding through the HD3 site.

EMSA using the DB1 probe also exhibited the formation of the specific DNA-protein complexes with both Arix and NBPhox (Fig 2.3D). With His-Arix, as the concentration of protein increases, two DNA-protein complexes are evident, a lower and an upper band. To ascertain whether the upper and lower complexes correspond to single or dual site occupancy at two homeodomain protein recognition sites, His-Arix was incubated with HD1m and HD2m probes, which contain mutations at either HD1 or HD2 sites within the DB1 oligonucleotide (Fig 2.3B). EMSA with the HD1m and HD2m probes resulted in the disappearance of the upper band, leaving the formation of only the lower band. Furthermore, a mutant DB1 probe in which both HD1/2 sites are disrupted by minimal nucleotide substitutions is unable to form DNA-protein complexes with both Arix and NBPhox (data not shown). These results indicate that the DNA-protein

complexes are HD site-specific and that the lower and upper DNA-protein complexes represent single occupancy at either the HD1 or HD2 site and dual occupancy at both the HD1/2 sites, respectively. Interestingly, the DNA-protein complex formed by NBPhox and the DB1 probe differs from the Arix-DB1 complexes, in that only one complex is evident, at the position of dual site occupancy (Fig 2.3D). The use of the HD1m or HD2m probes confirmed that a complex migrates to the position of single site occupancy when only one homeodomain site is present. Therefore, NBPhox appears to have a preference for dual, rather than single, site occupancy at the HD1/2 sites. This observation suggests cooperative binding of NBPhox to the HD1 and HD2 sites possibly through homodimerization. Further evidence that cooperative binding occurs at the DB1 oligonucleotide is the observation that the total binding activity of 3 units of NBPhox to the mutant HD1m and HD2m oligonucleotides is only 58% and 44% of the binding activity, respectively, of the oligonucleotide containing both sites intact (Fig 2.3D).

We also examined whether the CRE/AP1 element adjacent to the HD1 site affects the binding of Arix and NBPhox to DB1. EMSA with a probe containing a mutation in the CRE/AP1 site and leaving the HD1/2 sites intact produced a similar binding pattern with the wild type DB1 probe, demonstrating that binding of Arix and NBPhox to DB1 is independent of the CRE/AP1 site.

The above experiments (Fig 2.3C and 2.3D) demonstrated that both Arix and NBPhox can directly bind to the DBH promoter though the HD1, HD2, and HD3 sites. A quantitative representation of the EMSAs is presented in Fig 2.3E, where the total amount of bound DNA is plotted against the protein concentrations of recombinant Arix or NBPhox. The binding curves of Arix and NBPhox are nearly identical for both the

DB1 and the ATTA oligonucleotides, suggesting that each factor binds to the DB1 and the ATTA oligonucleotides with similar binding parameters. In addition, samples containing both Arix and NBPhox bound to an extent approximately equal to that of each factor individually (data not shown), demonstrating that binding parameters are not altered when both factors are present.

To evaluate whether the binding of Arix to the HD1 and HD2 sites of the DB1 oligonucleotide is cooperative, the fraction of probe in single site or dual site occupancy was analyzed as a function of Arix concentration (Fig 2.3F). Using the equation for cooperativity measurement described by Wilson *et al.* (165), the cooperativity factor τ was calculated at 50% of free probe (see Material and Methods for the equation). τ is defined as K_{d2}/K_{d1}, where K_{d1} and K_{d2} are dissociation constants for the reactions to fill the first binding site and to fill the second binding site, respectively. When τ value is more than 1, a cooperatively interacting binding system is implied. Therefore, τ describes the strength of cooperativity, the extent to which the binding of first site enhances the binding of the second site. In the case of Arix binding to DB1, at 50% of free DB1 probe, the population of singly liganded and doubly liganded DB1 is 18% and 23%, respectively; thereby, the doubly liganded DB1 is a predominant species of bound form of DB1. The average τ value for Arix from two experiments is 16.6. In comparison, the τ values for the paired-type homeodomain transcription factors Paired (165), Alx4 and goosecoid (157), are estimated at 65, 300, and 20, respectively. These experiments were performed using an ideal synthetic oligonucleotide, where the homeodomain binding sites are 3 bases apart. The τ value for Arix suggests moderate cooperativity in the binding system to HD1/2 sites within the DB1.

Arix and NBPhox may undergo homo- and heterodimerization.

The pattern of binding by both Arix and NBPhox suggests cooperative interactions between proteins and/or DNA, which leads to enhanced binding in the presence of two available sites. To examine whether Arix and NBPhox undergo protein-protein interactions, we developed an *in vitro* "His pull-down" assay with his-tagged recombinant proteins. In this assay, His-Arix or His-NBPhox are conjugated to metal affinity resin, TALON, and incubated with *in vitro* translated ^{35}S-labeled (IVT) proteins. Protein-protein interactions are assayed by the ability of IVT-proteins to be coprecipitated with His-Arix or His-NBPhox, and interacting proteins are resolved by SDS-PAGE. IVT-proteins used in these experiments were Arix and NBPhox as well as c-fos, a leucine zipper transcription factor which is structurally unrelated to the homeodomain proteins, and Herstatin, an extracellular portion of the receptor-like tyrosine kinase receptor, HER2 (Fig 2.4A) (26). The results of these experiments demonstrated that both ^{35}S-Arix and ^{35}S-NBPhox precipitate with His-Arix or His-NBPhox (Fig 2.4B). Neither ^{35}S-cfos nor ^{35}S-Herstatin were precipitated with either protein. No proteins were precipitated when resin was not conjugated with his-tagged proteins. These experiments suggest specific protein-protein interactions between Arix and NBPhox, leading to both homo- and heterodimer formation.

To control for the possibility that these protein-protein interactions are non-specifically mediated by association with DNA, the precipitates were treated with micrococcal nuclease prior to SDS-PAGE analyses. Micrococcal nuclease will digest any DNA adventitiously present in the assay mixtures, thus destroying any non-specific

DNA-mediated protein-protein interactions. The extent of interaction of ^{35}S-Arix and ^{35}S-NBPhox with His-Arix and His-NBPhox was diminished following nuclease treatment, but still evident (Fig 2.4C). These results demonstrate DNA-independent interactions between Arix and NBPhox molecules, and suggest that a portion of the EMSA results, especially preference for dual occupancy by Arix and NBPhox at the HD1/2 sites, can be attributed to these interactions. From these results it appears that homoprotein interaction occurs to the same extent as heteroprotein interaction.

Arix and NBPhox similarly regulate the DBH promoter.

To evaluate the regulatory influence of Arix and NBPhox on the DBH promoter, expression constructs in pcDNA3.0, containing either Arix (CMV-Arix) or NBPhox (CMV-NBPhox), were co-transfected with the DBH reporter plasmids DBH-Luc(-232) or DBH-CAT(-232) into two different noncatecholaminergic cell lines, hepatoma HepG2 and embryocarcinoma NT2. Both reporter constructs encompass 232 bases of the promoter and 5'-flanking sequences of the DBH gene, containing the critical enhancer region (DB1) as well as three putative homeodomain-binding sites (HD1, HD2, and HD3), presented in Figure 3A. Neither HepG2 nor NT2 cells express Arix and NBPhox (unpublished observation); therefore, their transcriptional activities can be directly examined without the effects of endogenously expressed Arix and NBPhox.

In order to examine the transcriptional activities of Arix and NBPhox, increasing amounts of Arix or NBPhox expression plasmids were introduced into HepG2 and NT2 cells. Two cell lines from different origins were used to assure that the responses observed with Arix and NBPhox are not specific to a certain cell type. In both cell lines,

basal transcription from the DBH promoter is increased to a maximum of 3-6 fold when Arix or NBPhox is co-transfected (Fig 2.5A). Transcriptional activities of Arix and NBPhox are concentration dependent and similar to each other. We have previously reported that the modest stimulation of DBH transcription with Arix is substantially increased with simultaneous stimulation of the PKA pathway (150). To evaluate whether NBPhox also interacts with the PKA system, expression plasmids containing NBPhox and PKA (RSV-PKA) were introduced into cells. Transfection of the DBH-reporter plasmid with the PKA expression vector causes stimulation of the DBH promoter 25 and 5 fold over basal activity in HepG2 and NT2 cells, respectively (Fig 2.5B). The concentrations (5-25 ng) of CMV-Arix or CMV-NBPhox, which evoke two-fold or less of an increase in the DBH-promoter activity when added alone (Fig 2.5A), now increase the reporter activity by 16-180 fold with co-transfection of PKA (Fig 2.5B). Thus, Arix and NBPhox result in synergistic stimulation of transcription with PKA from the DBH promoter in noncatecholaminergic cell lines.

To evaluate whether Arix and NBPhox, synergize or antagonize with each other to affect the DBH promoter, we introduced the same amounts of Arix and NBPhox expression constructs together into cells at submaximal plasmid concentrations (25 or 50ng each). The combination of both factors increased DBH activity to an extent equal to either factor alone under either basal or PKA-stimulated conditions (Fig 2.5C and 2.5D, respectively). These results demonstrate that Arix and NBPhox do not exhibit a synergistic interaction with each other; rather they act independently on the DBH promoter.

Characterization of functional domains of Arix

To further understand the mechanisms of Arix and NBPhox regulation of the DBH gene, we have initiated studies to define the domains of Arix responsible for regulatory activity. Truncated constructs of an HA-tagged Arix cDNA were produced, lacking either or both the N- or C-terminal regions of Arix (Fig 2.6A). These cDNAs encoding the truncated proteins were then co-transfected with the DBH-luciferase construct into either HepG2 or NT2 cells and the resultant transcriptional regulatory activities were examined under unstimulated and PKA-stimulated conditions. In the absence of PKA, the ArΔN construct resulted in a 50 and 66% reduction in the DBH-promoter activity as compared to the full-length Arix construct in HepG2 and NT2 cells, respectively (Fig 2.6B). The ArΔC construct exhibited transcriptional regulatory activity comparable to the full-length Arix in NT2 cells, while in HepG2 cells, ArΔC exhibited greater activity than wild type Arix, suggesting the loss of an inhibitory domain in the C-terminal segment of Arix. The construct containing only homeodomain, ArHD, has minimal ability to activate the DBH promoter.

In the presence of PKA, DBH promoter activity was activated by ArΔC to an extent similar to full-length Arix. In contrast, the transcriptional activities of ArΔN or ArHD constructs were substantially lower than that observed with full-length Arix in both cell lines tested. Their activities were approximately equal to the effect of PKA alone on the DBH-promoter (Fig 2.6B). These results suggest that the N-terminal segment (aa1-83) of Arix is a transactivation domain for both unstimulated and PKA-stimulated DBH transcription and that the Arix homeodomain itself does not mediate

DBH promoter activation. This result is in contrast to results with another paired homeodomain transcription factor, Phox1, which contains 72% amino acid identity with Arix and NBPhox within the homeodomain. With Phox1, the homeodomain alone is able to mediate transcription of the c-fos promoter, demonstrating that the activation domain resides in the homeodomain (45).

To establish that these truncated constructs were expressed and appropriately localized to the nucleus in mammalian cells, exogenously expressed proteins were detected by immunocytochemistry. The Arix construct was co-transfected with a EGFP expression construct (pEGFP) into cell cultures to identify transfected cells. One day after transfection, cells were prepared for immunohistochemical staining and analyzed by HA-antibody, followed by the detection based on either rhodamine fluorescence (Fig 2.6C) or HRP reactivity using diaminobenzidine (Fig 2.6D). For full-length, ArΔC and ArΔN constructs, fluorescent signals from rhodamine are readily visible, and localized to the nucleus, in contrast to the total cellular localization of EGFP. All cells that exhibit a fluorescent rhodamine signal also exhibit the green fluorescence indicative of EGFP expression, demonstrating the specificity of the antibody reaction with the epitope tagged Arix constructs. However, the construct containing only the homeodomain, ArHD, exhibits much fainter rhodamine fluorescence as well as diaminobenzidine staining. Similar results are observed when extracts from transfected cells are examined by western blot (data not shown). The lower expression level of the ArHD construct may be indicative of lower stability of this shortened protein *in vivo*. Nevertheless, these results establish that the truncated constructs are, in fact, expressed and appropriately localized to nucleus.

The results from the deletion analyses and the immunohistochemical detection of the truncated constructs indicate that the N-terminal segment (aa 1-83) of Arix is essential for both unstimulated and PKA-stimulated transcriptional activities of Arix and that the homeodomain itself does not carry the transactivation domain for DBH transcription *in vivo*. Since Arix and NBPhox have high amino-acid sequence homology in this N-terminal domain, NBPhox may also contain the functional domain in the corresponding region of the N-terminus and transactivate the DBH promoter.

DISCUSSION

The goal of the studies reported here was to compare the genetic organization and biochemical aspects of the transcriptional regulatory and DNA binding activities of a homeodomain protein, NBPhox, with that of the related protein, Arix. Phox2a/Arix and Phox2b/NBPhox have identical homeodomains and are both expressed in noradrenergic cell types. We have found that gene structures of Arix and NBPhox are similar each other although the genes are located on different chromosomes. Biochemical studies demonstrate that Arix and NBPhox bind to the same multiple target sequences in the DBH promoter and that they have similar binding affinity. In addition, homo- and/or heteroprotein interaction of these factors may mediate cooperative binding to the target DNA sequences. Our findings also show that Arix and NBPhox behave similarly and independently to regulate the transcription of the rat DBH promoter and exhibit the same concentration dependent transcriptional regulatory activity. Similar to Arix, NBPhox can also interact with the PKA pathway and synergistically increase transcriptional activity. Furthermore, we demonstrate that the region of these proteins bearing the greatest similarity, the N-terminal domain, is necessary for Arix transcriptional regulatory activity.

Arix and NBPhox share similar gene structures and different genomic locations.

Elucidation of the gene structure of Arix and NBPhox further delineates their similarity in origin, as well as that of several other genes encoding paired-like homeodomain proteins (11, 34, 142). Despite extensive diversity in the regions outside

of the homeodomain, and dispersion of the genes throughout the genome, these genes have maintained the similar 3 exon gene structure, with the exon/intron junctions occurring at the exact position within the homeodomain.

The genomic locations of mouse and human *Pmx2b* lie within regions of conserved linkage between these two species. Recently, using in situ hybridization, Yokoyama *et al.* reported that human PMX2B is located on Chromosome 5, instead of the Chromosome 4 location we have identified (172). Our results in mapping human PMX2B were consistent using two different mapping panels. The independent localization of the mouse and human homologues to a region of conserved linkage homology further supports our results.

Genetic mapping can often reveal linkage of a new gene to a previously mapped genetic disorder. Homeodomain genes are frequently associated with developmental disorders, caused by loss or gain of gene function. In one instance, found in the HOXD13 gene, an expansion of a region encoding polyalanine caused syndactly (105). Since NBPhox/Phox2b also contains a polyalanine tract, it could be associated with a similar genetic disorder. A search for human genetic disorders or mouse mutants in the regions of human Chromosome 4p12 and mouse Chromosome 5 produced the mouse neurological mutant pirouette, *pi.* (24). The phenotype of this mutant includes circling behavior, degeneration of the inner ear and hearing loss. Recently, the *pi* mutation has been mapped to within a 0.3 cM region of mouse Chromosome 5, proximal to *Gabrb1* and distal to *Recc1* (75). Since *Pmx2b* maps within this critical region, it can be considered a candidate gene for the *pi* mutant. However, we have sequenced the entire coding sequence of the *Pmx2*b gene in the *pi* mouse and did not detect a mutation (data

not shown). Therefore, if NBPhox is associated with *pi*, the mutation is in the non-coding region of the gene. In the human genome, near the locus of the PMX2B there is evidence for linkage to genes affecting vulnerability to alcohol dependence in a Southwestern American Indian tribe (91). Thus, while there is no direct association of Phox2b with a genetic disorder, subsequent linkage studies may reveal relationships.

Arix as well as NBPhox regulate DBH transcription similarly and independently.

Our results on the regulation of basal DBH transcription by Arix and NBPhox are consistent with those reported by Yang *et al.* using the human DBH promoter (170). Our studies have extended understanding of the regulatory properties of these proteins by our demonstration that both Arix and NBPhox interact with the PKA signaling pathway and form homo- and heterodimers. Finding that Arix and NBPhox function similarly and independently suggests that these two factors may have interchangeable roles *in vivo*. In the sympathetic ganglia, both factors are expressed throughout development, suggesting functional redundancy in the regulation of the neurotransmitter biosynthetic enzyme genes. In *Phox2a* -/- mice, Phox2b/NBPhox is still expressed in the sympathetic ganglia, and would have the ability to maintain the continual expression of DBH and TH (104). Experiments performed in our studies demonstrate the biochemical feasibility that Phox2b/NBPhox could substitute for the deleted Phox2a to regulate DBH transcription. From these experimental results, it would be predicted that any functional specificity distinguishing these closely related proteins would arise from differential patterns of gene expression, not from differential target gene selection within the same cells.

In previous studies, we demonstrated a synergistic interaction between Arix and PKA in the activation of the DBH promoter, involving the binding of AP1 transcription factors to the AP1/CRE site of the DB1 enhancer (149, 150). In the present study, we have found that NBPhox also interacts with the PKA pathway, resulting in a synergistic transcriptional response. Both Arix and NBPhox augment the response to PKA at a concentration whereby they alone have little effect on basal DBH transcription. This response suggests that Arix and/or PKA may be attracting other transcription factors, such as AP1, to the DBH promoter. Alternatively, activation of the PKA pathway may lead to phosphorylation of Arix and NBPhox, resulting in an increase in the transcriptional response. The importance of the synergistic interaction between Phox2a and PKA is also demonstrated in studies using mammalian neural crest stem cells (88). In this study, forced expression of Phox2a elicited expression of the TH gene only in cultures treated with forskolin, an activator of adenylate cyclase. Augmentation of Arix and NBPhox transcriptional activity by simultaneous stimulation of PKA may be an important event *in vivo*. Environmental signals encountered during the migration of neural crest cells leads to activation of intracellular second messenger systems such as the PKA pathway, which, in turn, interacts with tissue-specific homeodomain transcription factors such as Arix and NBPhox, resulting in the potent expression of DBH and consequently, the specification of neurotransmitter phenotype of neural crest cells.

Arix and NBPhox may share a similar mechanism to activate DBH transcription through the N-terminal segment.

In most cases, closely related homeodomain proteins bear no similarity to each other in regions outside of the homeodomain, raising questions concerning the role of the remaining amino acid sequence. Arix and NBPhox, however, bear 50% amino acid identity in the region N-terminal to the homeodomain. This N-terminal region (aa1-83) is necessary for transcriptional activity of Arix; therefore, it is plausible that the transactivation domain of NBPhox may also reside in the N-terminus. The functional interchangeability of these two family members and the homology within the putative activation domain suggests that they may function through a common molecular mechanism.

Identification of Arix activation domain is quite different in comparison to the functional domain of Phox1, a homeodomain transcription factor that has 72% identity to Arix only in the homeodomain. Phox1 imparts serum-responsive transcriptional activity to the serum responsive element (SRE) in the c-fos gene by interacting with serum responsive factor (SRF) (45). Unlike Arix, the homeodomain of Phox1 is a minimal functional domain, eliciting both DNA-binding and transcriptional activities. Alanine scanning mutagenesis suggests that helices 1 and 2 of the homeodomain are involved in making contacts with serum response factor, thereby promoting the recruitment of other accessory proteins such as Elk1, while helix3 functions as a DNA recognition helix (143). In contrast, our functional domain analysis of Arix suggests that the Arix homeodomain conveys only DNA-binding activity and serves to position the N-terminal activation domain to the target gene promoter, allowing for interactions with components

of the general transcription machinery to activate transcription of the DBH gene. This same N-terminal segment functions as an activation domain in a mammalian two hybrid assay, and interacts with the transcriptional co-activator, CBP, to potentiate transcription from the DBH-promoter in a PKA-dependent manner (148).

The N-terminal segment (aa1-83) necessary for transactivation of Arix does not reveal significantly high sequence homology to other proteins. However, we have recently identified a short peptide stretch (aa61-75), Brachyury-like motif, which has relatively high homology to a member of T-box gene family, Brachyury, and the paired-box protein, Pax9 (148). Although the Brachyury-like motif seems to be responsible for neither DNA binding nor transcriptional activity in Brachyury (17, 73) and Pax9 (112), it may serve as a critical structural motif to orient the transactivation and the DNA-binding domains of transcription factors.

The possible role of dimerization of Arix and NBPhox in the DBH gene regulation

Arix and NBPhox are members of the paired type homeodomain family. Several paired type homeodomains have been found to exhibit cooperative interactions upon binding to a synthetically selected target containing two homeodomain recognition sites (157, 165). One of the important factors in the cooperativity is the spacing between two recognition sites. The strongest cooperativity was shown when two recognition sites are separated by three bases (P3). As the number of spacer nucleotides is increased or decreased, the strength of cooperativity is diminished, and a spacer greater than five nucleotides abolishes cooperativity. In our study, we have evaluated the binding and activation properties of Arix and NBPhox with the naturally occurring dual

homeodomain recognition sites in the DB1 enhancer. In the DB1 enhancer, the HD1/2 sites are separated by six bases and binding of protein to both HD1/2 sites would be predicted to be non-cooperative. However, despite the six base spacing between HD1/2 sites, binding of Arix to both sites of the DB1 enhancer exhibits moderate cooperativity. Arix may achieve cooperativity in DNA binding with the assistance of homodimerization, which was demonstrated in the *in vitro* interaction studies. In contrast, the paired homeodomain proteins Alx4 and Cart-1, which exhibit cooperative binding to the P3 site, do not exist as dimers in solution and do not exhibit direct physical protein-protein interactions (121, 157). One difference between our experiments and those of others is that we are using the full-length proteins, rather than only homeodomains, in both our EMSA and interaction studies. The *in vitro* homodimerization of Arix and NBPhox may occur through a protein-protein interaction domain residing outside of the homeodomain. If homodimerization is an obligatory step for cooperative binding, this binding may not have been observed in experiments using only the homeodomain. The homodimeriztion of Arix and NBPhox may influence the activation of DBH gene transcription by facilitating binding to the dual homeodomain sites of the DB1 enhancer. This interpretation is consistent with our transactivation analyses, where the homeodomain of Arix, which would not be predicted to exhibit cooperative binding, does not itself function as a transcriptional activator.

The NBPhox-NBPhox interaction is apparently strong enough that binding to the DB1 enhancer is only observed as dual site occupancy. Since NBPhox binds only as dual occupancy, we are unable to evaluate whether the parameters of Arix binding is altered when both proteins are present. However, the total amount of binding to either a single

or dual recognition site is the same when both Arix and NBPhox are included in the EMSA as when either protein is evaluated separately (data not shown).

Other structurally related paired-type homeodomain proteins, Alx4 and Cart1, have been shown to exhibit overlap in biological function (121). Alx4 and Cart1 contain 92% amino acid identity within the homeodomain but divergent sequences outside the homeodomain. These proteins possess similar DNA binding activity on the synthetic P3 oligonucleotide and transactivate a promoter containing multiple copies of the P3 site. Similar to Alx4 and Cart1, both Arix and NBPhox equally transactivate the DBH promoter, and the combined presence of both proteins does not alter the extent of promoter activation. The functional redundancy of these similar paired type homeodomain proteins may have evolved to assure appropriate transcription of critical genes, as well as to allow subtle differences in the patterns of target gene expression.

Acknowledgments

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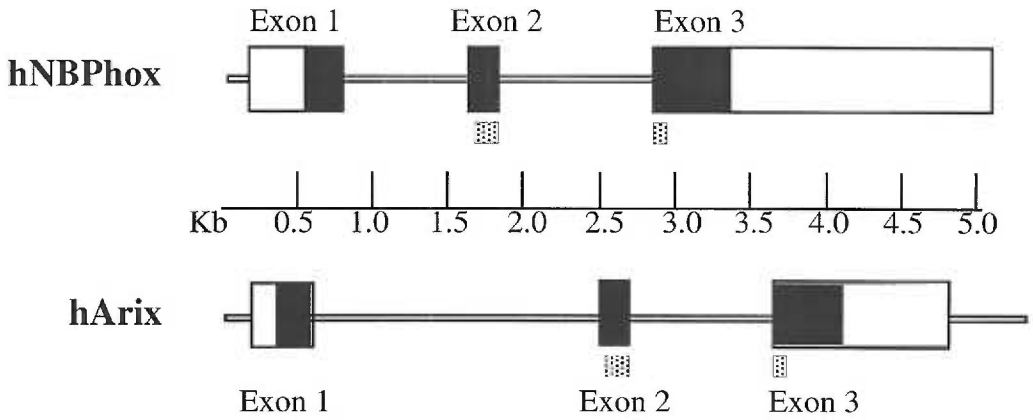
Figure 2.1. Gene structure of human Arix and NBPhox are similar. A. A schematic diagram comparing the exon/intron structure of the human genes encoding NBPhox and Arix is shown. The blackened regions represent coding sequence, while the shaded boxes below each gene indicate the position of the homeodomain. **B.** A comparison of the amino acid homology between the human Arix and NBPhox genes, divided by exon, is shown.

Figure 2.1

(A)



(B)

Exon 1

```
hArix     ---MDYSYLN S--YDSCVAA MEASAY---- GDFGACSQPG GFQYSPLRPAFPAAGPPCPA LGSSNCALGA LRDHQPAPYS AV
hNBPhox   MYKMEYSYLN SSAYESCMAG MDTSSLASAY ADFSSCSQAS GFQYNPIRTTFGAT-SGCPS LTPGSCSLGT LRDHQSSPYA AG
```

Exon 2

helix3 (recognition helix)

```
hArix     PYNFFPEPSG LHEKRKQRRI RTTFTSAQLK ELERVFAETH YPDIYTREELALKIDLTEAR VQ
hNBPhox   PYKLFTDHGG LNEKRKQRRI RTTFTSAQLK ELERVFAETH YPDIYTREELALKIDLTEAR VQ
```

homeodomain

Exon 3

```
hArix     VWFQNRRAKF RKQERAASAK GAAGAAGAKK GEARCSSEDD DSKESTCS-PTPDSTASLPP PPAPG-LASP RLSPSPLPVA LGSGPGP-GP
hNBPhox   VWFQNRRAKF RKQERAAAAA AAAAKNGS-S GKKSDSSRDD ESKEAKSTDPDSTGGPGPNP NPTPSCGANG GGGGGPSPAG APGAAGPGGP
```

Exon 3

```
hArix     GPNPLKG--- ALWAGVAGGG GGGPGAGAAE LLKAWNPAE- --SGPGPF-- ---------SGVLSSFHRKP G--PALKTNL F
hNBPhox   GGEPGKGGAA AAAAAAAAAA AAAAAAAAGG LAAAGGPGQG WAPGPGPITS IPDSLGGPFGSVLSSLQRPN GAKAALVKSS M
```

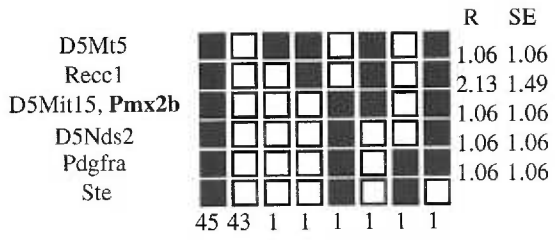
Figure 2.2. NBPhox is located on mouse Chromosome 5 and human Chromosome 4p12. A. Haplotype figures from The Jackson BSB and BSS backcrosses showing part of Chromosome 5 with loci linked to *Pmx2b*. Loci are listed in order with the most proximal at the top. The black boxes represent the C57BL6/JEi allele and the white boxes the SPRET/Ei allele. The number of animals with each haplotype is given at the bottom of each column of boxes. The percent recombination (R) between adjacent loci is given to the right of the figure, with the standard error (SE) for each R. Missing typings were inferred from surrounding data where assignment was unambiguous. **B.** The map location of mouse *Pmx2b*. The linkage relationships in the region of chromosome 5 where *Pmx2b* maps are shown for the BSS backcross panels. Map figure from The Jackson BSS backcross showing part of Chromosome 5. The map is depicted with the centromere toward the top. A 3 cM scale bar is shown to the right of the figure. Loci mapping to the same position are listed in alphabetical order. Missing typings were inferred from surrounding data where assignment was unambiguous. Raw data from The Jackson Laboratory were obtained from the World Wide Web address http://www.jax.org/resources/documents/cmdata **C.** The map location of human PMX2B. Relative positions of markers were derived from both the GeneBridge 4 mapping data and the gene map developed by the International RH Mapping Consortium, found at internet address www.ncbi.nlm.nih.gov/genemap/. The comparison between the radiation hybrid and cytogenetic maps is derived from the latter internet site. The comparative map between human and mouse genomes has been developed and maintained by Dr. Michael Seldin, University of California, Davis (23), and updated information is found at URL: www.ncbi.nlm.nih.gov/homology/. G4=GeneBridge 4

radiation hybrid panel; G3= Stanford 3 radiation hybrid panel.

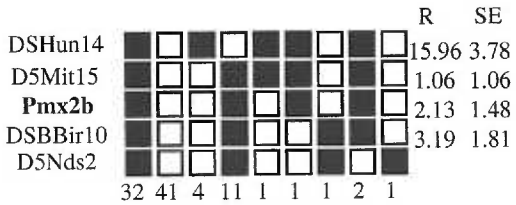
Figure2.2

(A)

Jackson BSS Chromosome 5



Jackson BSB Chromosome 5



(B)

Jackson BSS Chromosome 5

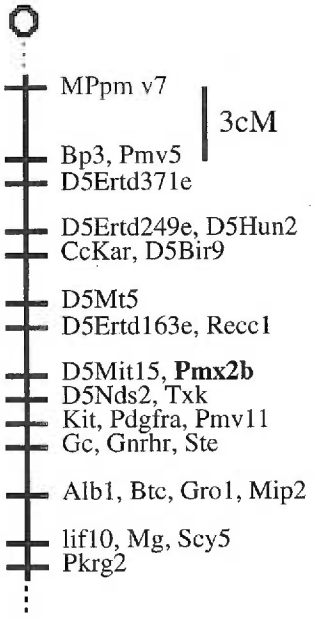


Figure 2.2

(C)

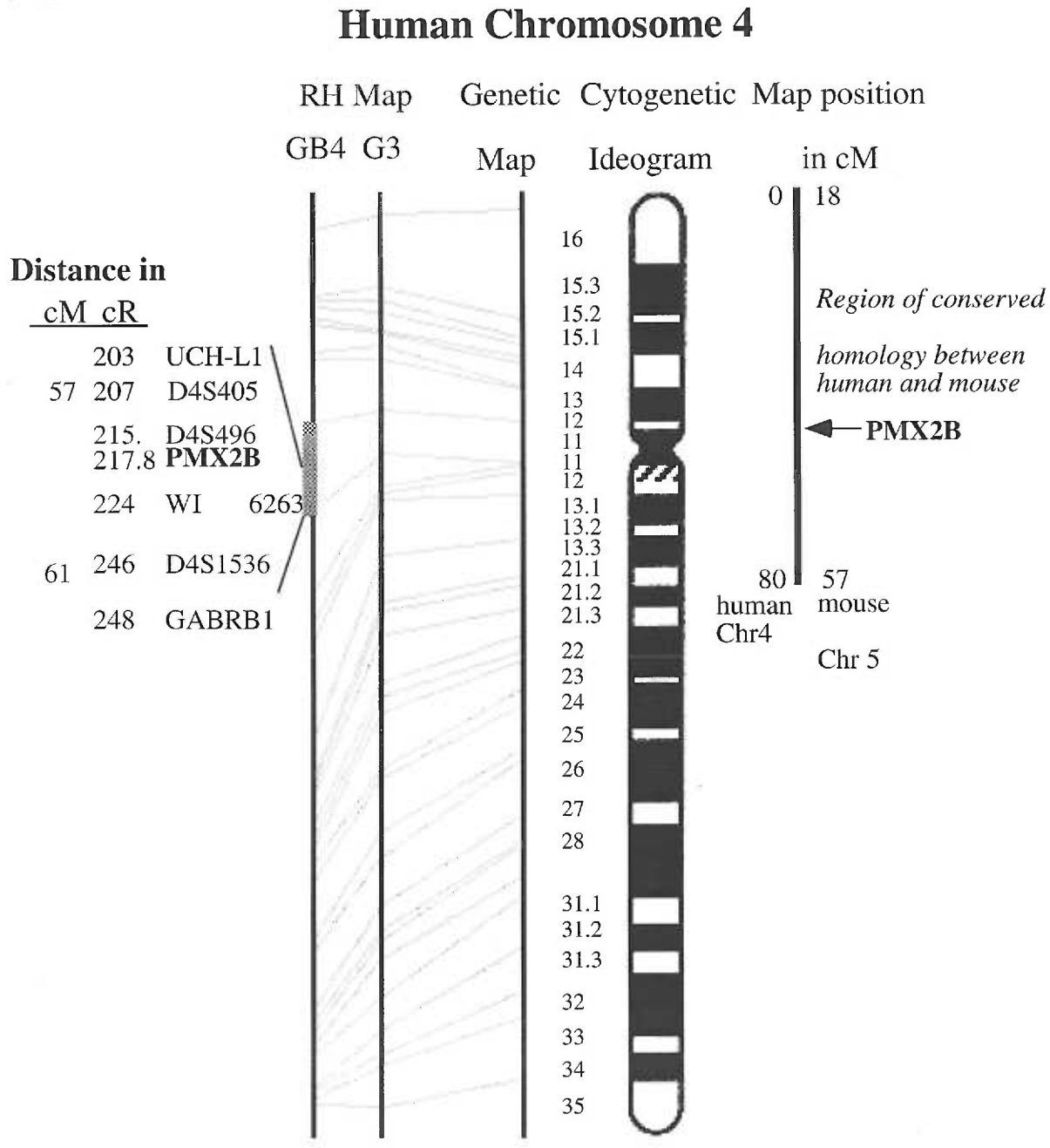
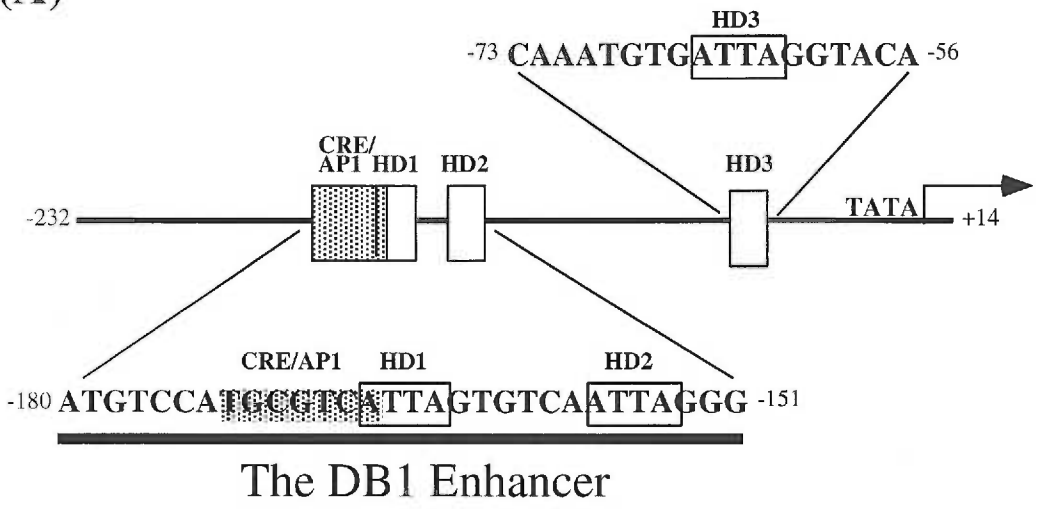

Human Chromosome 4
RH Map
GB4 G3
Genetic Map
Cytogenetic Ideogram
Map position in cM
Distance in
cM cR
203 UCH-L1
57 207 D4S405
215. D4S496
217.8 PMX2B
224 WI 6263
61 246 D4S1536
248 GABRB1
16
15.3
15.2
15.1
14
13
12
11
11
12
13.1
13.2
13.3
21.1
21.2
21.3
22
23
24
25
26
27
28
31.1
31.2
31.3
32
33
34
35
0
18
Region of conserved
homology between
human and mouse
PMX2B
80
human
Chr4
57
mouse
Chr 5

Figure 2. 3. Both Arix and NBPhox bind to the DBH promoter through homeodomain protein binding sites. A. A schematic diagram of the rat DBH promoter (-232/+14) with a sequence detail of the regulatory elements, CRE/AP1 (shaded), HD1, HD2, and HD3 (white boxes) sites. **B.** Wild-type oligonucleotides, DB1 and ATTA, and mutant oligonucleotides, HD1m, HD2m, HD3m and CRE/AP1m, used in EMSA are depicted. **C.** Increasing amounts of recombinant His-Arix or His-NBPhox were incubated with the ATTA probe, bearing the HD3 site, in the binding reaction of EMSA. An unlabeled ATTA probe was also used to compete proteins interacting with the ATTA probe. Arix and NBPhox did not form DNA-protein complexes with the HD3m probe in which the HD3 site is disrupted. One unit of Arix or NBPhox contains approximately the same amount of protein. **D.** The binding of Arix and NBPhox to the DB1 oligonucleotide was analyzed by EMSA. The DB1 oligonucleotide was incubated with increasing amounts of recombinant Arix or NBPhox. For competition EMSA, unlabeled DB1 was used as indicated. Mutant oligonucleotides, HD1m and HD2m, were also used as a probe to compare the Arix and NBPhox binding patterns with DB1 probe. White and black arrow heads indicate single and dual occupancy of DB1 probe by His-Arix or His-NBPhox, respectively, through the HD1 and HD2 sites. Both Arix and NBPhox formed DNA-protein complexes with the CRE/AP1m oligonucleotide similar to those with DB1. **E.** The extent of the binding ability of His-Arix or His-NBPhox to the ATTA and the DB1 probes were quantitatively analyzed based on EMSA results. Free and bound probes (DNA-protein complexes) were quantitated using a phosphoimager. Fractions of total bound probes are expressed in percentages and plotted against the concentrations of His-Arix or His-NBPhox by units. Circles and squares represent Arix and NBPhox,

respectively. The solids lines indicate the best fit of the Hill equation obtained as described in Materials and Methods. The parameter values for each curve are: A_{max}=79.4, K_x=2.2, h=2.1, A_0=-0.95 in Arix-ATTA; A_{max}=80.9, K_x=1.9, h=2, A_0=1.1 in NBPhox-ATTA; A_{max}=92.7, K_x=1.8, h=1.8, A_0=0.03 in Arix-DB1; A_{max}=82.3, K_x=1.5, h=1.9, A_0=1.6 in NBPhox-DB1. **F.** The cooperativity of Arix binding to the DB1 was analyzed from EMSA. Fractions of free probe (squares), singly occupied DB1 (circles), and doubly occupied DB1(triangles) are quantitated and plotted against the concentrations of Arix. The data points are fitted to the Hill equation as described in Materials and Methods. The parameter values are: A_{max}=-0.9, K_x=7, h=2.3, A_0=1 in free probe, A_{max}=0.29, K_x=6.3, h=2.8, A_0=0 in singly occupied DB1; A_{max}=0.85, K_x=11, h=1.6, A_0=-0.02 in doubly occupied DB1.

Figure 2.3

(A)


HD3
-73 CAAATGTGATTAGGTACA -56
CRE/
AP1 HD1 HD2
HD3
-232
TATA
+14
CRE/AP1 HD1 HD2
-180 ATGTCCATGCGTCATTAGTGTCAATTAGGG -151
The DB1 Enhancer

(B)

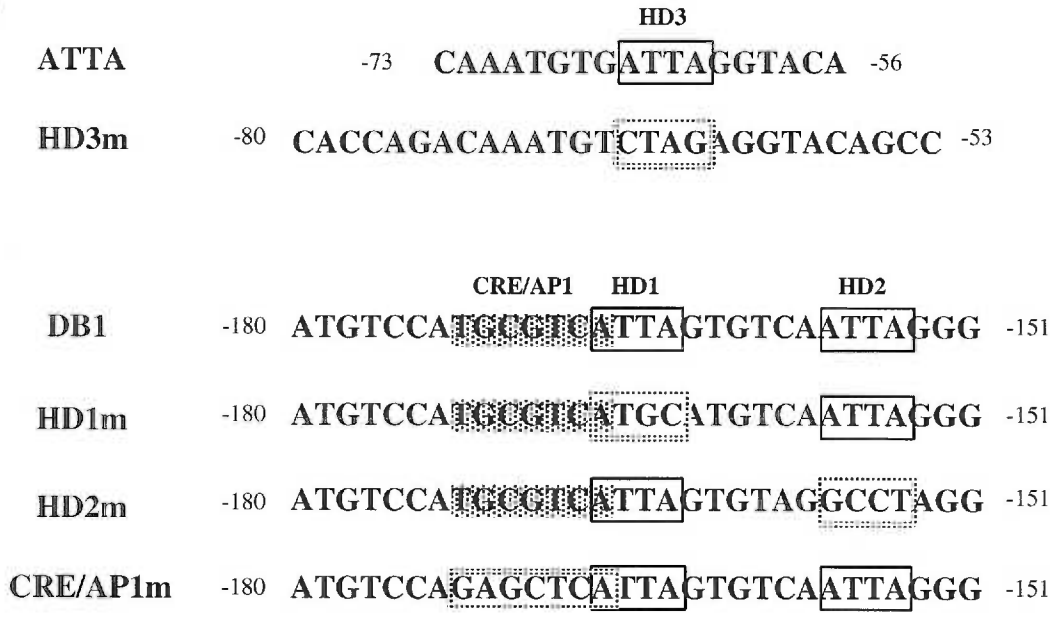
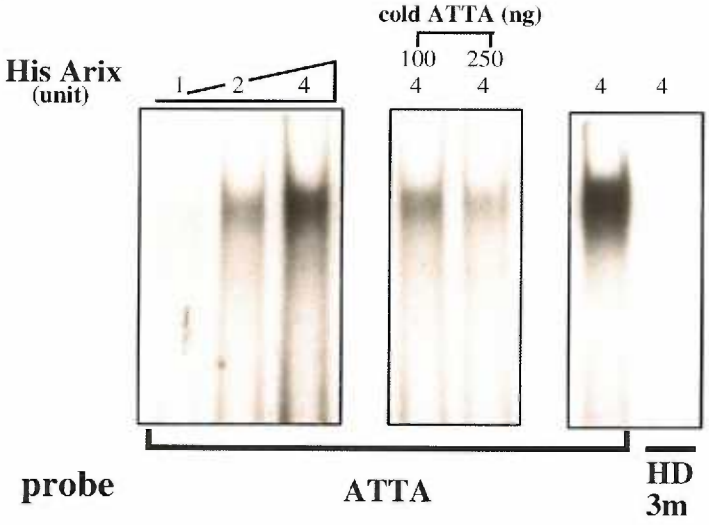

HD3
ATTA -73 CAAATGTGATTAGGTACA -56
HD3m -80 CACCAGACAAATGTCTAGAGGTACAGCC -53
CRE/AP1 HD1 HD2
DB1 -180 ATGTCCATGCGTCATTAGTGTCAATTAGGG -151
HD1m -180 ATGTCCATGCGTCATGCATGTCAATTAGGG -151
HD2m -180 ATGTCCATGCGTCATTAGTGTAGGCCTAGG -151
CRE/AP1m -180 ATGTCCAGAGCTCATTAGTGTCAATTAGGG -151

Figure 2.3

(C)


cold ATTA (ng)
100 250
His Arix
(unit)
1 2 4
4 4
4 4
probe
ATTA
HD
3m

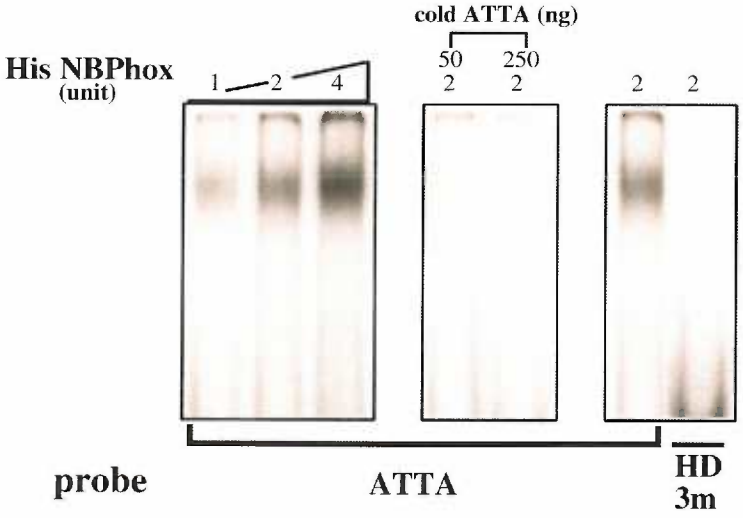

cold ATTA (ng)
50 250
His NBPhox
(unit)
1 2 4
2 2
2 2
probe
ATTA
HD
3m

Figure 2.3

(D)

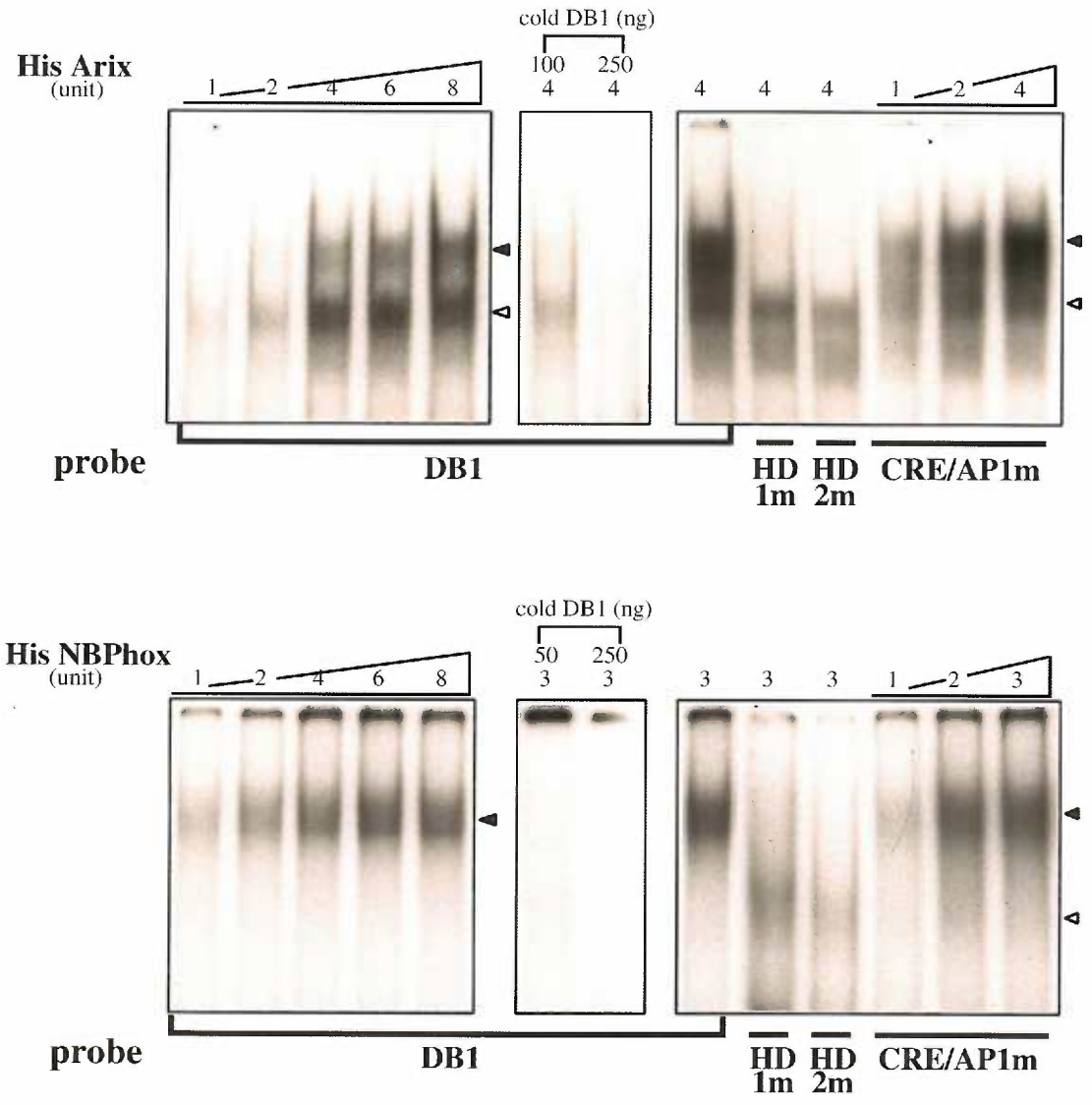
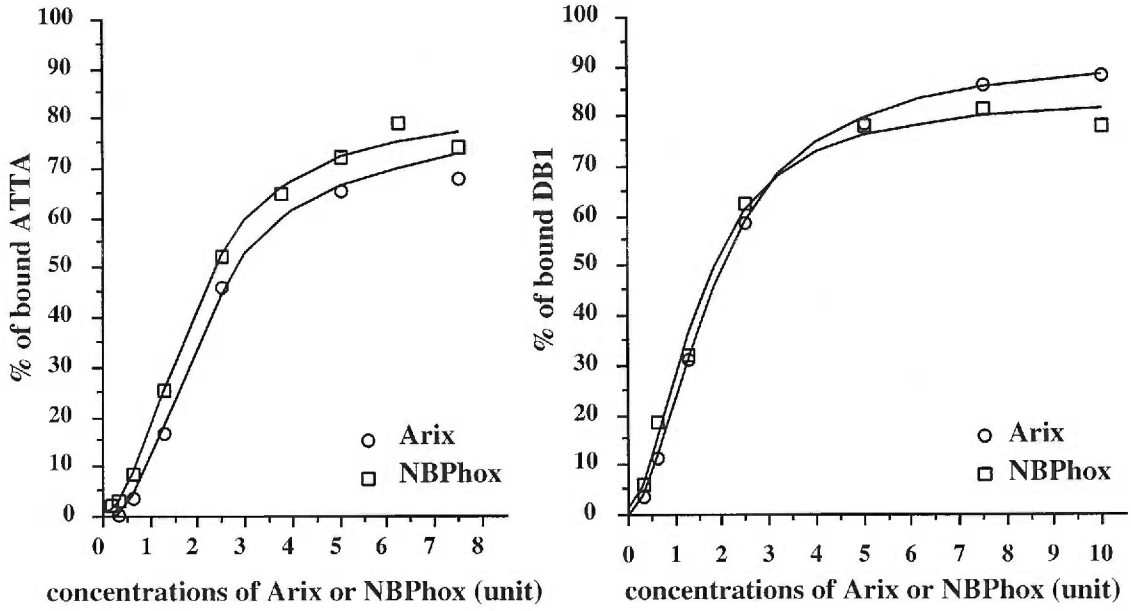

cold DB1 (ng)
His Arix
(unit)
1 2 4 6 8
100 250
4 4
4 4 4
1 2 4
probe
DB1
HD 1m
HD 2m
CRE/AP1m
cold DB1 (ng)
His NBPhox
(unit)
1 2 4 6 8
50 250
3 3
3 3 3
1 2 3
probe
DB1
HD 1m
HD 2m
CRE/AP1m

Figure 2.3

(E)


% of bound ATTA
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8
Arix
NBPhox
concentrations of Arix or NBPhox (unit)
% of bound DB1
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10
Arix
NBPhox
concentrations of Arix or NBPhox (unit)

(F)

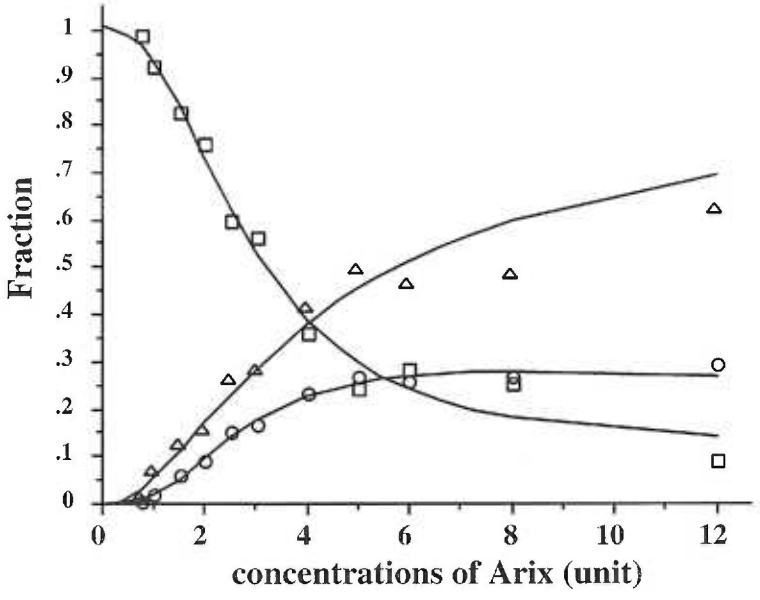

Fraction
1
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
0 2 4 6 8 10 12
concentrations of Arix (unit)

Figure 2.4. Arix and NBPhox can form homo and hetero oligomers. A. *In vitro* translated and ^{35}S-labeled (^{35}S-IVT) Arix , NBPhox, cfos, and Herstatin (HST) were resolved on SDS-PAGE, followed by autoradiography. **B.** ^{35}S-IVT proteins were incubated with TALON metal resin either in the presence or the absence of recombinant His-Arix and His-NBPhox. After the binding reactions, resin was washed as described in Materials and Methods. His-Arix or His NBPhox interacting proteins were analyzed by SDS-PAGE and detected by ^{35}S signals. **C.** After the same *in vitro* binding assays described above, washed resin was further treated with micrococcal nuclease prior to SDS-PAGE analyses. His-Arix and His-NBPhox interacting proteins are evidently present although overall ^{35}S signals are weaker than in micrococcal nuclease -untreated reactions. The binding assays with and without micrococcal nuclease treatment were carried out in parallel and examined by autoradiography with an exactly same exposure time.

Figure 2.4

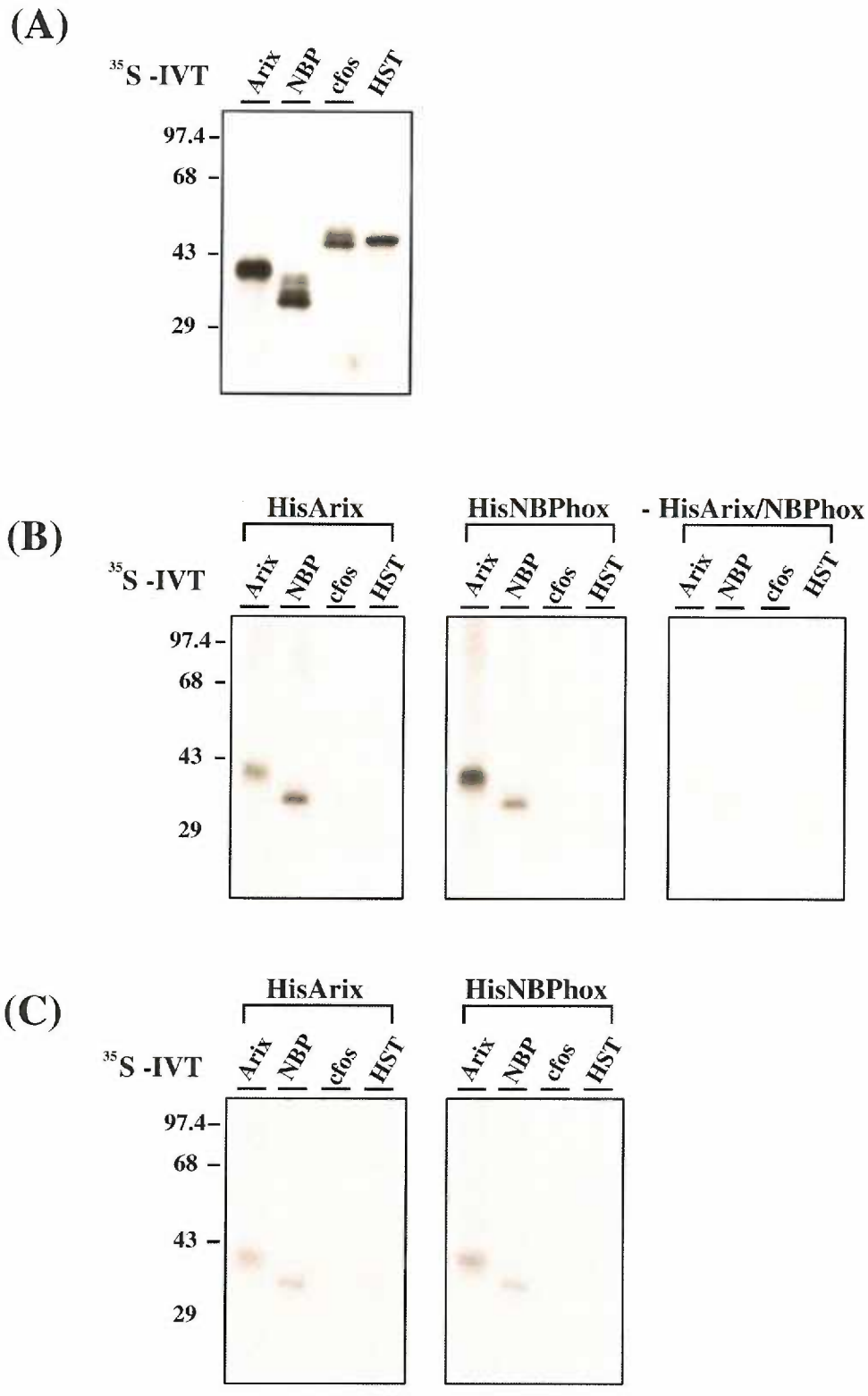
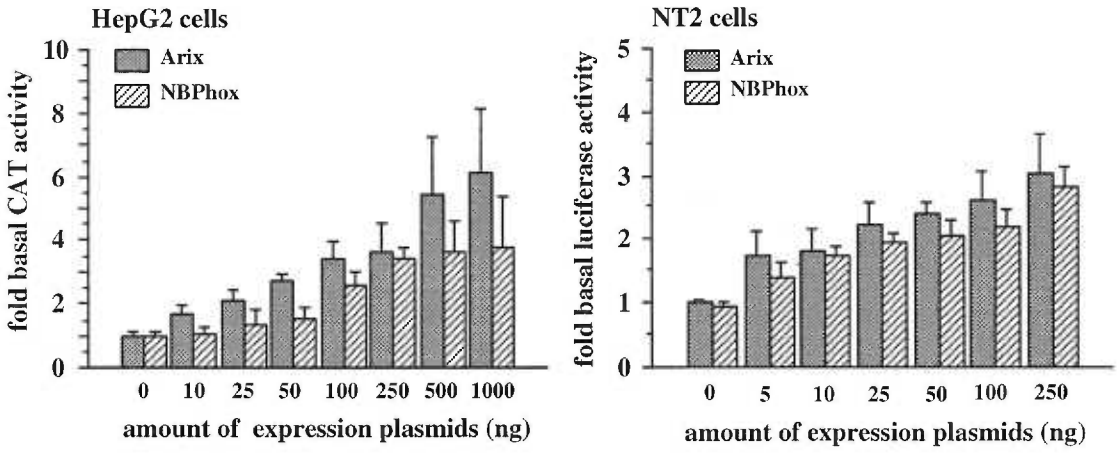

(A)
35S -IVT
Arix
NBP
cfos
HST
97.4
68
43
29
(B)
HisArix
HisNBPhox
- HisArix/NBPhox
35S -IVT
Arix
NBP
cfos
HST
97.4
68
43
29
(C)
HisArix
HisNBPhox
35S -IVT
Arix
NBP
cfos
HST
97.4
68
43
29

Figure 2.5. Transcriptional activities of Arix and NBPhox on the DBH promoter. HepG2 cells were transiently transfected with 5 μg of DBH-Luc, 333 ng of pRL-CMV (a *Renilla* Luciferase plasmid, Promega), increasing amounts of either Arix or NBPhox expression plasmid, cloned in vector pcDNA3.0, and either in the absence (A) or in the presence (B) of 2.5 μg of RSV-PKA by a calcium phosphate precipitation method. For NT2 cell transfection, 500 ng of DBH-Luc, 33 ng of pRL-CMV, and increasing amounts of either Arix and NBPhox expression vectors were used either without (A) or with (B) 250 ng of RSV-PKA. Cells were harvested 24 hr or 48 hr after transfection and subjected to CAT or luciferase assays. CAT and luciferase activities were normalized to protein concentrations in cell lysate. Normalized CAT or luciferase activities are expressed relative to the activity from the basal control group, where a backbone of expression vector (HA6.1) was transfected in the absence of PKA. Thus, the mean values of the fold CAT or luciferase activity for the basal control are standardized to 1. 4-6 representative transfections from three independent experiments done in triplicate were selected to analyze the data. The bars express the mean + S.E. Similarly, DBH-CAT and DBH-Luc activities were examined in HepG2 and NT2 cells, respectively, when expression vectors of Arix and NBPhox were co-transfected either each construct alone or together as indicated in either the absence (C) or the presence (D) of RSV-PKA. CAT and luciferase activities were normalized to protein concentrations. The data represent 4-6 representative transfections from three independent experiments done in triplicate. The bar express the mean + S.E.

Figure 2.5

(A) -PKA



(B) +PKA

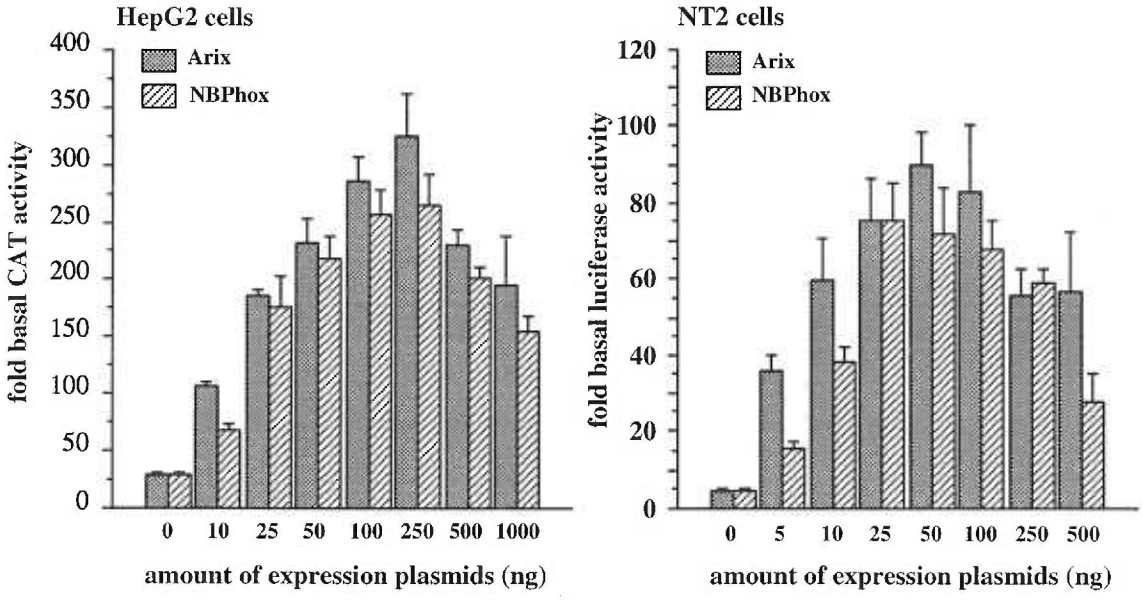
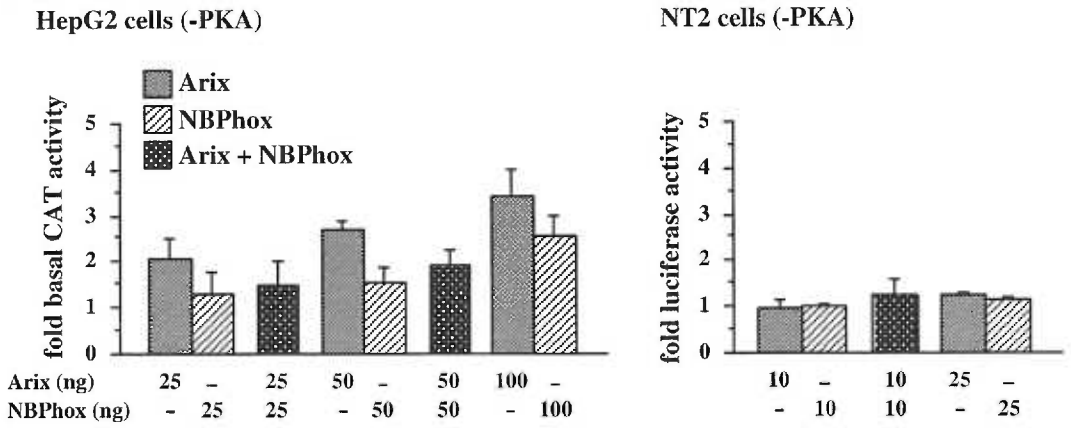


Figure 2.5

(C)


HepG2 cells (-PKA)
NT2 cells (-PKA)
Arix
NBPhox
Arix + NBPhox
fold basal CAT activity
fold luciferase activity
0
1
2
3
4
5
Arix (ng) 25 – 25 50 – 50 100 –
NBPhox (ng) – 25 25 – 50 50 – 100
10 – 10 25 –
– 10 10 – 25

(D)

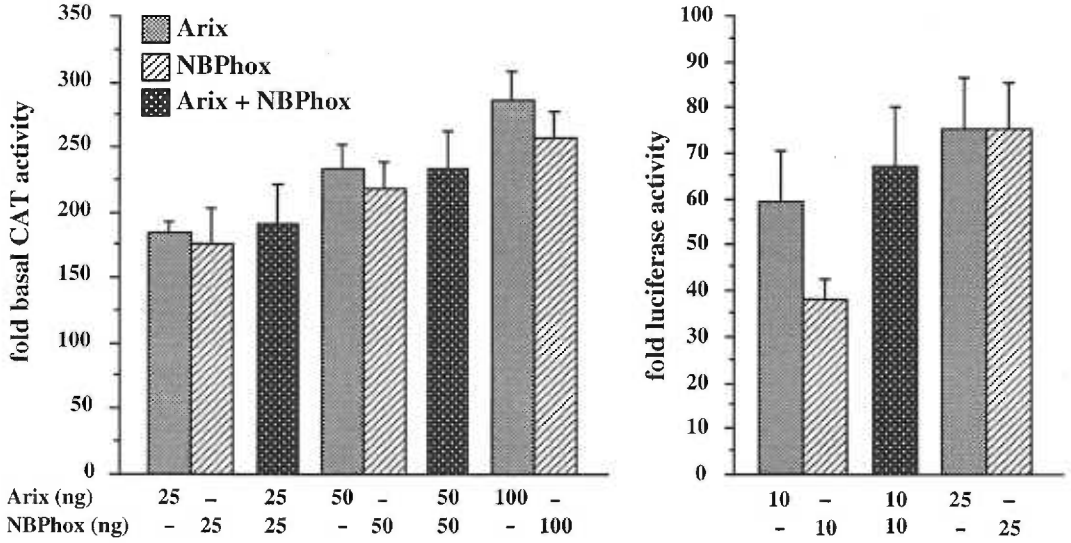

HepG2 cells (+PKA)
NT2 cells (+PKA)
Arix
NBPhox
Arix + NBPhox
fold basal CAT activity
fold luciferase activity
0
50
100
150
200
250
300
350
10
20
30
40
50
60
70
80
90
100
Arix (ng) 25 – 25 50 – 50 100 –
NBPhox (ng) – 25 25 – 50 50 – 100
10 – 10 25 –
– 10 10 – 25

Figure 2.6. The N-terminus of Arix is critical for both basal and PKA mediated transcriptional activities of Arix. A. A schematic diagram of truncated Arix constructs used for transfections. All constructs were fused to a HA epitope tag at the N-terminus, designated by a black box in the figure. Numbers in parentheses of each construct correspond to amino acid residues of Arix. **B.** NT2 cells were transiently transfected with 1 μg or 25 ng of a given truncated or full-length Arix construct along with 500 ng of DBH-Luc, 33 ng of pRL-CMV, and either with or without 250 ng of RSV-PKA using lipids as described in Methods and Materials. HepG2 cells were transiently transfected with 2 μg or 50 ng of a given truncated or full-length Arix construct along with 5 μg of DBH-Luc, 333 ng of pRL-CMV, and either with or without 2.5 μg of RSV-PKA by a calcium phosphate precipitation method. Cells were harvested 24 hr after transfection and assayed for luciferase activities and protein concentrations. Luciferase activities were normalized to protein concentrations in cell lysates. Normalized luciferase activities are expressed relative to the activity from the basal control group, where a backbone of Arix expression vector (HA6.1) was transfected in the absence of PKA; thus, the mean value of the fold luciferase activity for the basal control is standardized to 1. The data in NT2 cell transfection are based on 4-6 representative transfections from three independent experiments and are expressed as the mean + S.E. In HepG2 cell transfection, results from a representative experiment are presented and bars represent the mean + S.E. These analyses were done in triplicate in each transfection and repeated with similar results. **C.** HepG2 cells were transiently co-transfected with 100ng of pEGFP and 100ng of a given truncated or full-length Arix construct. 24hr after transfection, cells were fixed and stained with HA antibody. HA signals were detected

by rhodamine based fluorescence. The expression of EGFP indicates transfected cells. Representative examples of transfected cells were shown in phase contrast, EGFP, and rhodamine epifluorescent illumination of the same microscopic fields. Objective magnification is 40X. **D.** HepG2 cells transiently transfected with 100 ng of a given truncated or full-length Arix were stained with HA antibody. HA signals were detected by HRP reaction using diaminobenzidine. Objective magnification is 40X.

Figure 2.6

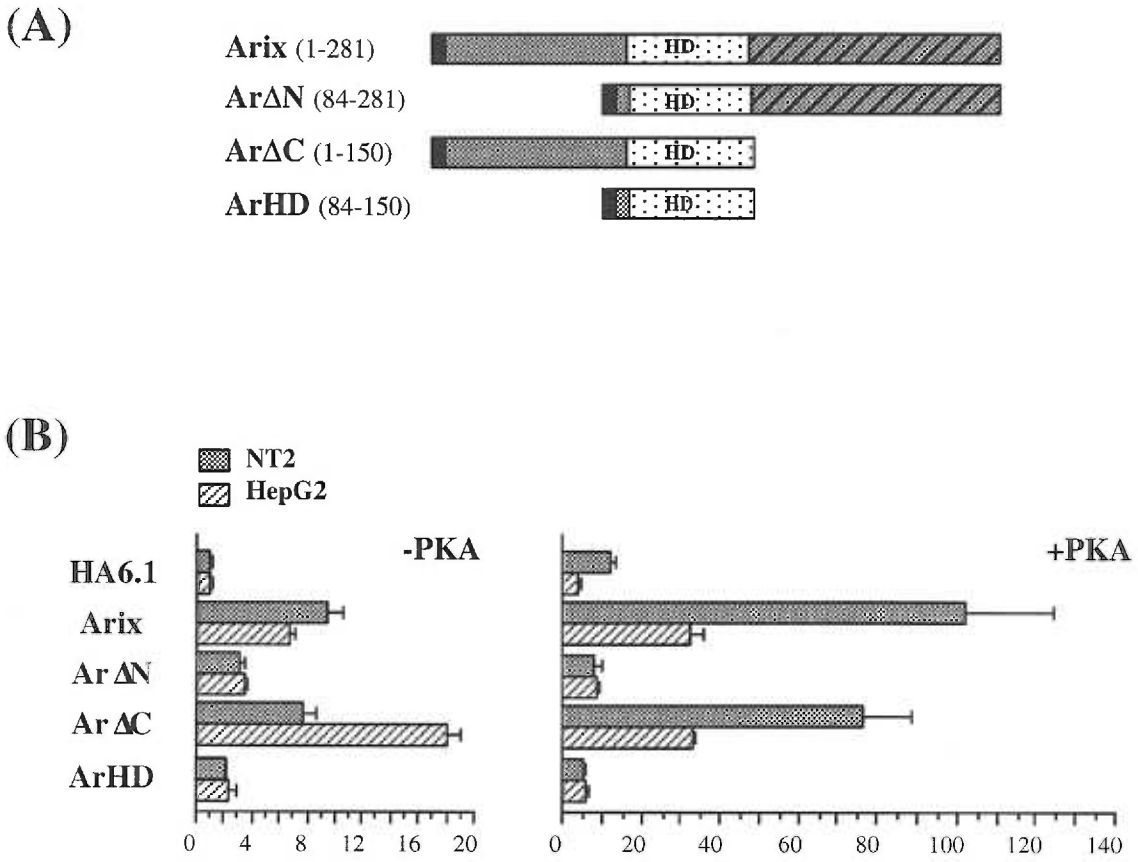
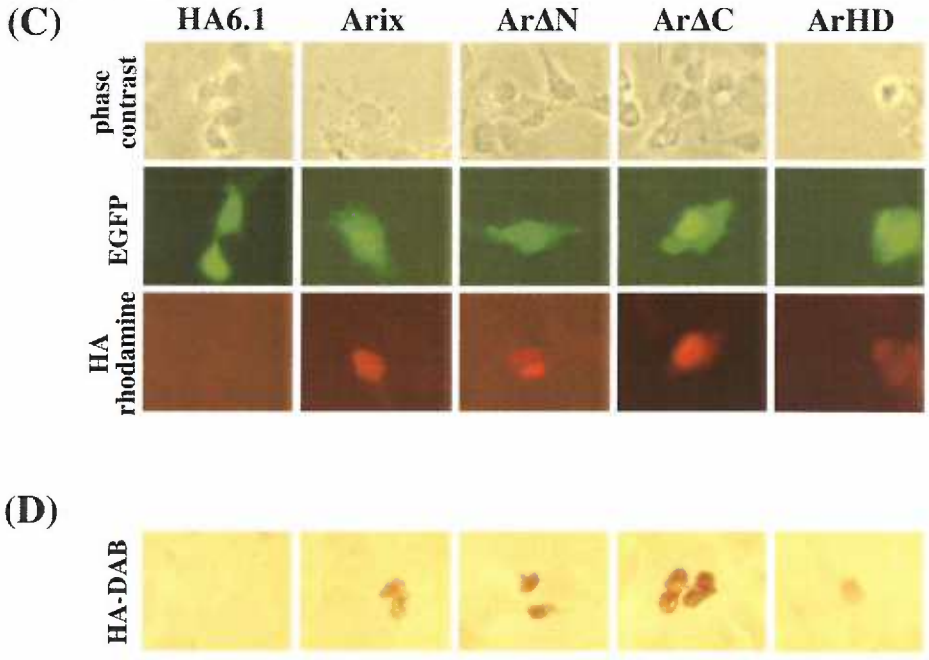

(A)
Arix (1-281)
ArΔN (84-281)
ArΔC (1-150)
ArHD (84-150)
HD
(B)
NT2
HepG2
-PKA
+PKA
HA6.1
Arix
Ar ΔN
Ar ΔC
ArHD
0 4 8 12 16 20
0 20 40 60 80 100 120 140
Fold basal luciferase activity

Figure 2.6


(C)
HA6.1
Arix
ArΔN
ArΔC
ArHD
phase contrast
EGFP
HA rhodamine
(D)
HA-DAB

Chapter Three

The Paired-Like Homeodomain Protein, Arix, Mediates Protein Kinase A-stimulated Dopamine ß-Hydroxylase Gene Transcription Through Its Phosphorylation Status

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Running tittle

The role of Arix phopshorylation in DBH transcription

ABSTRACT

The homeodomain transcription factor, Arix/Phox2a, plays a critical role in the specification of noradrenergic neurons by inducing the expression of dopamine ß-hydroxylase (DBH), the terminal enzyme for noradrenaline biosynthesis. In reporter assays, Arix together with activation of cyclic AMP (cAMP)-dependent kinase (PKA) potentiates DBH gene transcription. We have evaluated whether post-translational modification of Arix regulates PKA-mediated DBH gene transcription. We found that Arix is constitutively phosphorylated *in vivo* at the basal level and that the phosphorylation level was substantially decreased upon stimulation of the PKA pathway. The change in the Arix phosphorylation state coincides with DNA-binding activity of Arix. Treatment of cells with forskolin results in a robust enhancement of Arix's DNA-binding, which is reversed by serine/threonine and tyrosine phosphatase inhibitor treatments. Consistent with DNA-binding activity of Arix, following tyrosine phosphatase inhibitor treatment, the DBH promoter activity mediated by Arix and PKA is reduced by 40%. The results collectively suggest that dephosphorylation of Arix is a necessary event to fully activate PKA-mediated DBH transcription. Thus, the present study demonstrates that Arix can integrate extrinsic signals through post-translational modification, regulating the DBH gene transcription in response to activation of the PKA pathway.

INTRODUCTION

Homeodomain proteins are involved in the determination of cell-type specificity in a variety of organisms. In the central and peripheral nervous systems, identity of neurotransmitter is a pivotal attribute for proper function of a neuron. Neurotransmitter phenotype is represented by the ability of a neuron to synthesize, release, and uptake the neurotransmitter. Therefore, the expression of a biosynthetic enzyme and/or vesicular transporter in its given neuron defines a neurotransmitter identity. A coordinated array of gene expression ultimately defines the phenotype of any neuron. Indeed, many transcription factors have been shown to play a key role in deciding the fate of a progenitor cell and in linking the cell to a variety of extracellular stimuli during development.

Noradrenergic neurons are characterized by the coexpression of the catecholamine biosynthetic enzymes, tyrosine hydroxylase (TH) and DBH. TH, the rate-limiting enzyme of catecholamine biosynthesis, catalyzes the production of DOPA, which is in turn converted to dopamine by amino acid decarboxylase. DBH is the terminal enzyme that produces noradrenaline from dopamine; thus, the expression of DBH is essential for determination of noradrenergic cells. For specification of noradrenergic neurons, the paired-like homeodomain transcription factors, Arix/Phox2a and NBPhox/Phox2b, appear to act in concert to regulate noradrenergic traits in both the central and peripheral nervous systems (116). Arix and NBPhox are closely related homeodomain proteins that share significant amino acid sequence homology, including 100% identity in the homeodomain and 50% identity in the N-terminus to the

homeodomain. Coordinate action of Arix and NBPhox is essential for the proper development of central and peripheral noradrenergic cells. Targeted deletion analyses of Phox2a (104) and Phox2b (117) demonstrate the necessity of these genes to direct noradrenergic neuronal differentiation. Despite this implication, forced expression of Phox2a is only able to induce the expression of TH, but not DBH in mammalian neural crest stem cell cultures. Importantly, the expression of both TH and DBH is evoked by Phox2a only together with bone morphogenetic protein 2 (BMP2) and forskolin, which increases an intracellular cAMP level (88). In contrast, in the avian embryo culture, forced expression of Phox2a is sufficient to promote the generation of ectopic noradrenergic neurons that express TH, DBH, and pan-neuronal genes (145). These experimental findings *in vivo* and *in ovo* suggest that Phox2a requires an additional factor and/or an environmental stimulus that is present in embryo in order to potentiate activation of target genes.

In addition to the genetic manipulation studies, we and others have demonstrated a direct link between Phox2 transcription factors and DBH gene expression by analyses of the 5' upstream promoter of the DBH gene (150) (1) (69). In previous studies, we found three homeodomain protein recognition sites (HD1, 2, and 3) for Arix and NBPhox within the proximal rat DBH promoter. Reporter assays using mutant promoters showed that all three HD sites are integrated and interdependent to regulate DBH transcription. Indeed, both Arix and NBPhox can directly activate transcription from the DBH promoter and can synergize with the cAMP/PKA pathway to produce a potent activation of the DBH transcription. They are effective at a similar protein concentration and elicit similar maximal responses, suggesting redundant roles of Arix and NBPhox in DBH gene

regulation as well as a link between transcription factors and extracellular stimuli. Furthermore, detailed dissection of the DBH promoter identified a genetic element, DB1, that mediates PKA responsiveness, noradrenergic tissue specificity, and Arix-dependent activation of DBH transcription (139) (150). DB1 is an enhancer composed of the cAMP-response element/activator protein 1 (CRE/AP1) site adjacent to a pair of HD sites (HD1/2). Enhancement of DBH transcription by second messengers occurs in catecholaminergic cell lines expressing endogenous DBH and is mediated through the CRE/AP1 element on the DBH promoter (149). We have also demonstrated that binding of AP1 family proteins, including cFos and cJun, to the CRE/AP1 site is necessary to achieve the transcriptional synergism of Arix with PKA pathway (149). In addition, recruitment of the coactivator, CRE-binding protein (CREB)-binding protein (CBP), appears to be involved in the DBH promoter activation by Arix and PKA (148). CBP physically interacts with the transactivation domain of Arix and augments Arix-mediated DBH transcription in a PKA-dependent manner. Thus, CBP is a functional component of the signal-dependent transcriptional network in the DBH gene transcription. However, it is unknown how Arix acts through multiple HD sites to integrate the activation of the PKA pathway. One plausible mechanism would involve post-translational modification of Arix in response to PKA activation.

Protein phosphorylation is an important post-translational modification to modulate the function of proteins in response to the extracellular stimuli. In general, the phosphorylation state of a transcription factor may influence its activity by modifying nuclear translocation, DNA binding, or transactivation potential (57, 163). A few cases of homeodomain proteins whose function is regulated by phosphorylation have been

reported. The Cut homeodomain transcription factor is phosphorylated *in vitro* by casein kinase II (CKII) and protein kinase C (PKC) in the Cut repeats, a DNA-binding motif in addition to the homeodomain, causing a reduction in DNA binding and transcriptional repression (19, 20). CKII is also known to phosphorylate the homeodomain of Csx/Nkx2.5, increasing DNA binding (65). PKA can directly phosphorylate the homeodomain of Oct-1 *in vivo* in a mitosis specific manner (136). Evidence of phosphorylation of homeodomain proteins further extends to functional relevance *in vivo*. Regulated phosphorylation of *Drosophila* Antennapedia by CKII plays an important role in appropriate development of thorax and abdomen during embryogenesis (60). These developmental influences appear to result from the ability of Antennapedia to bind DNA cooperatively with the other homeodomain protein, Extradenticle, upon phosphorylation of Antennapedia.

Natural target genes for most of the homeodomain proteins are unknown; therefore, little is known about the transcriptional mechanisms to achieve their functional specificity. In the present study, we took advantage of the known *in vivo* target gene for Arix, and have characterized the molecular mechanism of Arix in activation of DBH gene transcription in coordination with activation of the PKA pathway. We found that Arix is a constitutive phosphoprotein and that the phosphorylation state of Arix is dramatically decreased in response to activation of the PKA pathway. PKA activation leads to increased DNA-binding activity of Arix, which is abolished by pre-treatment with phosphatase inhibitors. Collectively, the present study suggests that a dephosphorylated form of Arix is transcriptionally active and that the regulated post-translational modification is a crucial event to functionally activate Arix in response to an extracellular

stimuli. This is the first demonstration on the functional regulation of a paired-type homeodomain protein by phosphorylation.

MATERIALS AND METHODS

Cell culture— HepG2 cells were cultured in minimum Eagle's medium (MEM) supplemented with 10% fetal bovine serum (Hyclone), 1% non-essential amino acids, and 110mg/l sodium pyruvate. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with an addition of 10% fetal bovine serum. Both cell lines were maintained at 37°C in an atmosphere of humidified air containing 5% CO_2.

Plasmid constructs— The construction of DBH-Luc (-232/+10) reporter plasmid containing the promoter and 5'-flanking sequence of DBH gene was described previously (148). Briefly, this reporter construct contains the proximal DBH promoter (-232/+10) including the DB1 enhancer region, TATA-like sequence, and the transcription start site, linked upstream of the coding sequence of firefly (*Photinus pyralis*) luciferase. The RSV-PKA expression construct, containing cDNA for the catlytic subunit of PKAα, was a generous gift from Dr. Richard Maurer [Oregon Health Sciences University, previously described by Maurer (96)]. Hemaglutinin (HA) -tagged full length (HA-Arix) and truncated Arix (ArΔN, ArΔC, and HDAr) expression plasmids were constructed as described by Adachi *et al.* (1). Point mutations of Arix constructs were made in HA-Arix expression vector by oligonucleotide directed mutagenesis (Quick Change, Stratagene). To create the Y25F construct, the complementary oligonucleotide pair 5'-GCCGAGACCCACttcCCGGACATTTACACTCG-3' (base pair substitutions in lower case) was designed where tyrosine residue is replaced with phenylalanine. The T30A construct was made using the complementary oligonucleotide pair 5'-

CGGACATTTACgctCGTGAGGAACTGGC-3' in which threonine reside is mutated to alanine.

Transfections and Reporter Assays— DNA used for transfections was purified by equilibrium centrifugation in $CsCl_2$-ethidium bromide gradients. For HepG2 cell transfections, cells were plated at a density of 0.8 x 10^6 cells per well in a 6-well plate one day before transfection and transfected with 2μg of DNA by calcium phosphate precipitation as described previously (Shaskus *et al.*, 1992). The total amounts of DNA were adjusted using HA6.1, the backbone of an expression vector. Cells were harvested 24 hr after transfection and aliquots of cell extracts were assayed for protein content and luciferase activity using Dual-Luciferase Assay System (Promega). For some experiments, cells were stimulated with forskolin at a concentration of 20μM for 7hr before harvest. Orthovanadate treatment (1mM at final concentration) was carried out for 30min prior to the forsklin stimulation. As an internal control of transfection efficiency, all transfections contained a jellyfish luciferase (Renilla) plasmid that lacks a promoter (pRL-null); thereby, the expression of Renilla is independent of a co-transfected PKA expression plasmid. Thus, values presented for reporter gene activity are standardized to Renilla luciferase activity per extract.

In Vitro Kinase Assays— For substrates, *in vitro* translated and ^{35}S-labeled Arix, truncated Arix constructs, and NBPhox were produced using TNT coupled wheat germ extract system (Promega). Phosphorylation of substrates were carried out at 30°C for 30min in an ATP-regenerating kinase buffer system. The kinase buffer includes 0.5μl of

in vitro translated, ^{35}S-labeled substrate, 50mM Tris-Cl (pH8), 2mM $MgCl_2$, 5mM ATP, 10mM phosphocreatine, 3.5u/ml creatine kinase, 2.5μM okadaic acid, 0.5mM sodium orthovanadate and either 10μg of cell nuclear extracts or 10ng of purified, recombinant catalytic subunit of PKA (rPKA) as a source of kinases. In some kinase reactions, 5μM of a PKA specific inhibitor, H-89, was included during incubation. In dephosphorylation experiments, 0.06 unit of potato acid phosphatase (Roche) was added in the kinase reaction mixture. The reaction products were analyzed by SDS-PAGE, followed by autoradiograph. For the *in vitro* kinase assay using [γ-^{32}P]ATP, bacterially produced and purified His-tagged Arix protein (1μg) (1) were incubated at 30°C for 5 min with 10ng of rPKA in the kinase reaction buffer containing 12.5mM Tris-Cl (pH8), 0.1mM ATP, 10mM $MgCl_2$, 0.25mg/ml BSA and 0.5μCi [γ-^{32}P]ATP. The reaction was stopped by adding EDTA at the final concentration of 80mM on ice. As control reactions, wild-type and mutant CREB proteins (0.5μg each) were used as substrates. The reaction products were separated on a SDS-PAGE gel and autoradiographed. rPKA and wild-type and mutant CREB proteins were generous gifts from Dr. Peter Rotwein (Oregon Health Sciences University).

In Vivo Phosphorylation Studies—3.5-4 x 10^6 of HEK293 cells were seeded on a 100mm polylysine-coated plate one day before transfection. 10μg of DNA was transfected using Lipofectamine (GIBCO/BRL) according to the manufacturer's instruction. The total amount of DNA was adjusted with HA6.1. The following day, cells were incubated in phosphate-free DMEM containing 500 μCi/ml of ^{32}P-orthophosphate for 4hr. Cells were harvested and lysed in RIPA buffer (50mM Tris-Cl

pH8, 150mM NaCl, 1mM EDTA, 0.5% deoxycholate, 1% NP-40, 0.1%SDS) and lysates were cleared by centrifugation. HA-tagged Arix was immunoprecipitated with HA-antibody (3F10, Roche) and resolved on 10% SDS-polyacrylamide gel, followed by autoradiography. For quantitation of ^{32}P incorporation, a gel was exposed to a Phosphoimager (Molecular Dynamics) and ^{32}P signals were quantitatively analyzed.

Two-dimensional phosphoamino acid mapping analyses— ^{32}P-labeled proteins were excised from SDS-PAGE gels and ground in 1ml of 50mM ammonium bicarbonate (pH7.3) solution including 10μl of 10% SDS and 50μl of ß-mercaptoethanol. The samples were boiled for 5min and eluted with shaking at room temparature for 3 hr twice. Eluted proteins were precipitated by 250μl of trichloroacetic acid on ice for 1hr with 40μg of RNaseA as a carrier protein and collected by centrifugation. Resultant pellets were washed with ice-cold 100% ethanol, air-dried and subjected to hydrolysis in 5.7M HCl for 1hr at 110 ˚C. After hydrolysis, samples were lypholized in a Speed-Vac and resuspended in 5-10μl of pH1.9 buffer (88% formic acid, acetic acid, and deionized water at a ratio of 50:156:1794 [vol/vol]), which contains phosphoamino acid standards. Samples were then electrophoresed on a cellulose thin-layer plate in the pH1.9 buffer at 1.5kV for 20min as the first dimention, followed by the second dimension electrophoresis in pH3.5 buffer consisting of acetic acid, pyridine, and deionized water (100:10:1890 [vol/vol]) at 1.3kV for 16 min. The plate was completely dried, sprayed with 0.25% ninhydrin solution in aceton to visualize the standards, and autoradiographed.

In Vitro Protein-Protein Interaction Assay— 5μg of soluble fractions containing bacterially expressed His-Arix or His-NBPhox fusion proteins were conjugated to TALON metal affinity resin (Clontech) and co-incubated with 3μl of *in vitro* translated ^{35}S-labeled proteins in 250μl of the binding buffer (10mM Tris-HCl, 200mM NaCl, 20mM imidazole, and 1mg/ml BSA adjusted to pH8) for 2hr at 4˚C. After the incubation, resin was washed three times with 1ml of the binding buffer containing 0.25% NP-40, followed by two additional washes with 1ml of the binding buffer without NP-40. The resin was boiled in SDS-PAGE sample buffer for 5min and subjected to SDS-PAGE, followed by autoradiography. In some experiments, the resin was further treated with micrococcal nuclease to eliminate DNA contaminants. The resin-protein conjugates were subsequently washed and resuspended in 50μl of digestion buffer (10mM Tris pH8, 50mM NaCl, and 4mM $CaCl_2$) after the protein-protein interaction assay described above. The resin was incubated with 0.4 unit of micrococcal nuclease at 37˚C for 1hr and washed with 1 ml of the digestion buffer and 1ml of the binding buffer without NP-40 prior to SDS-PAGE.

HEK293 Cell Extracts and Electrophoretic Mobility Shift Assays (EMSA)— To prepare cell extracts used for EMSA, HEK293 cells were plated at a density of 3.5-4 x 10^6 cells in a 100mm polylysine-coated dish and transfected with the Arix expression construct (10μg) next day using Lipofectamine (GIBCO/BRL) according to the manufacturer's instructions. During transfection, HEK293 cells were incubated in OPTIMEM (GIBCO/BRL) with a lipid-DNA mixture at a ratio of 3 to 1 for 5 hr. The following day, cells were treated with forsklin (20μM at a final concentration) or vehicle for 1hr before

harvest. Some of them were pre-treated with okadaic acid (50nM) or orthovanadate (1mM) for 30 min prior to the forsklin stimulation. Harvested cell pellets were resuspended in 80-100 μl of the RIPA buffer and incubated for 20min on ice. The cell extracts were collected by centrifugation and the protein concentration was assayed by Bradford (BioRad). To quantitate the amount of Arix in cell extracts, 0.5μg of cell extracts were separated on 10% SDS-polyacrylamide gel and subjected to western blot analyses using HA antibody (3F10, Roche).

For EMSA, synthetic sense and antisense oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and then annealed. EMSA were carried out in 20μl of the binding reaction buffer (12.5mM Hepes pH7.9, 5mM $MgCl_2$, 50mM KCl, 1mM EDTA, 1mM dithiothreitol, 2μg of poly(dI-dC)·poly(dI-dC), and 10% glycerol). Labeled probe (20,000cpm in Cerenkov counts) and 0.1μg of HEK293 cell extracts were added in the binding reaction followed by 20min incubation at room temperature. For the competition and supershift assays, the binding reactions were pre-incubated for 20min in the presence of 50ng of unlabeled double-stranded oligonucleotides or 0.5μg of HA antibody (3F10) (Roche), respectively, prior to the addition of labeled oligonucleotides. DNA-protein complexes were resolved on 6% non-denaturing polyacrylamide gels (19:1 = acrylamide : bisacrylamide) in electrophoresis buffer (45mM Tris borate and 1mM EDTA) followed by autoradiography. For the quantitation of EMSA, relative proportions of bound probes were calculated using a Phosphoimager (Molecular Dynamics).

RESULTS

Arix is post-translationally modified *in vitro* and *in vivo*. We have previously demonstrated that Arix can produce a modest activation of transcription from the rat DBH promoter, and that the transcriptional activity of Arix is greatly potentiated by stimulation of the PKA pathway (150). In previous studies, we found that PKA-dependent DBH transcription is partly mediated by recruitment of Fos and Jun to the CRE/AP1 element on the DBH promoter (149). Therefore, if Fos and Jun are the downstream targets of the PKA stimulation, we would anticipate that Fos and Jun would mimic the effect of PKA on the DBH transcription. To analyze this hypothesis, Fos and Jun expression counstructs were transfected with the DBH promoter-reporter construct plus Arix expression construct. Consistent with our previous observation, Arix activated the DBH promoter by 3 fold (Fig. 1) in HepG2 hepatoma cells, which lack the expression of endogenous Arix as well as DBH (data not shown). When cells were treated with forskolin, an activator of adenylate cyclase, to stimulate the PKA pathway, Arix greatly potentiated the DBH transcription by 14 fold. However, cotransfection of Fos and Jun induced the DBH transcription by only 4 fold with Arix, a value very similar to activation of transcription with Arix alone. Since the presence of Fos plus Jun was not sufficient to fully activate DBH transcription, we hypothesized whether a different mechanism such as post-translational modification of Arix, underlies PKA-stimulated DBH transcription. To address the question, we first tested whether Arix is phosphorylated *in vitro* (Fig. 2A). *In vitro* kinase assays were performed, using *in vitro* translated (IVT) and ^{35}S-labeled proteins and PC12 cell nuclear extracts as substrates and a source of kinases,

respectively. In this kinase assay, IVT-Arix and NBPhox were resolved on the SDS-PAGE and visualized by ^{35}S signals. We then looked for the mobility shifts that were eliminated by treatment of potato acid phosphatase as evidence of phosphorylated products. Addition of PC12 cell nuclear extracts resulted in multiple mobility shifts, which were sensitive to potato acid phosphatase, from IVT-Arix as well as NBPhox, implicating that both transcription factors can be phosphorylated by a kinase in PC12 nuclear extract (Fig.2A). Interestingly, the use of a PKA specific inhibitor, H-89, did not eliminate mobility shifts created by PC12 nuclear extracts in both Arix and NBPhox. Consistently, a direct application of recombinant PKA (rPKA) was unable to form mobility shifts. We also tested for direct incorporation of [γ-^{32}P]ATP into bacterially produced recombinant Arix by rPKA. rPKA efficiently phosphorylated recombinant CREB protein as demonstrated by ^{32}P incorporation, but a mutant form of CREB, which lacks PKA phosphorylation sites at serine 133 residue (Fig. 2B). Recombinant Arix exhibited no significant ^{32}P incorporation by rPKA. These results strongly suggest that the phosphorylation of Arix and NBPhox is not a direct action of PKA.

Next, we examined whether Arix is phosphorylated *in vivo* by metabolically labeling cells with ^{32}P-orthophosphate. The Arix cDNA construct was tagged with hemaglutinin (HA) antigen and HEK293 cells were transiently transfected with HA-Arix, followed by immunoprecipitation with an HA antibody. We chose HEK293 cells because they have a much higher transfection efficiency (approximately 80%) than HepG2 cells, which have only 2-3% transfection efficiency (Adachi and Lewis unpublished observation). Furthermore, similar to HepG2 cells, HEK293 cells lack endogenous expression of Arix and demonstrate the activation of the DBH promoter by

Arix and PKA; together Arix and the activation of PKA potentiated DBH transcription compared to Arix or PKA alone (Fig. 2C). When HEK293 cells were transiently transfected with HA-Arix expression vector and metabolically labeled with ^{32}P-orthophosphate, ^{32}P incorporation of Arix was apparent. This result is evidence that Arix is a constitutively phosphorylated protein (Fig. 2D). Importantly, PKA activation dramatically decreased ^{32}P incorporation of Arix by 80%. Western blot analysis demonstrated that the expression of Arix was equivalent between the basal and PKA-stimulated conditions.

Protein-protein interactions of Arix and NBPhox are favored in dephosphorylated states. Previously, we reported that Arix and NBPhox have the ability to form homo- and heterodimers *in vitro* (1) and *in vivo* (148). Protein-protein interactions between Arix and NBPhox at the multiple homeodomain recognition sites on the DBH promoter may be a mechanism to regulate DBH transcription. In order to seek the functional relevance of the phosphorylation event of Arix, we examined whether there is preference in either the phosphorylation or dephosphorylation state of Arix and NBPhox for protein-protein interactions. First, ^{35}S-labled IVT proteins were subject to the kinase reaction with PC12 nuclear extracts to generate phosphorylated proteins as described above (Fig. 3A). ^{35}S-labled IVT proteins containing phosphorylated products were then used for His pull-down assays. In these assays, protein-protein interactions were monitored by measuring the ability of ^{35}S-labeled IVT proteins to coprecipitate with His-Arix or His-NBPhox proteins conjugated to Ni resin. His-Arix or His-NBPhox interacting proteins were resolved on SDS-PAGE and detected by ^{35}S signals. His-Arix

interacting proteins were primarily the faster migrating proteins from both IVT-Arix and NBPhox, presumably dephosphorylated proteins (Fig. 3B). In contrast, an analysis of an aliquot of supernatant after the binding reaction demonstrated the presence of slower migrating proteins, suggesting that unbound fractions are mostly phosphorylated products. Consistently, in a reciprocal experiment using His-NBPhox conjugated Ni-resin, His-NBPhox interacting proteins were mostly in a dephosphorylated form of Arix and NBPhox. To eliminate the possibility that these protein-protein interactions are non-specifically mediated by association of DNA, the precipitates were further treated with micrococcal nuclease to digest non-specific DNA prior to SDS-PAGE analyses (Fig. 3C). Even after the micrococcal treatment, the protein-protein interactions were evident. These protein-protein interaction studies imply that dephosphorylated rather than phosphorylated Arix and NBPhox favorably undergo physical interactions.

PKA-dependent dephosphorylation of Arix may be a key step to activate the DBH transcription. Within the DBH promoter, there are three homeodomain recognition sites (HD1, 2, and 3) (Fig. 4A). Recombinant Arix and NBPhox can bind to the DBH promoter through the HD1, 2, and 3 sites (1), and all three sites are functionally required to regulate the DBH transcription (148). Based on our previous findings, we used oligonucleotides containing these sites, named DB1 and HD3 probes (Fig. 4A), to examine whether the DNA-binding activity of Arix is altered upon activation of the PKA pathway. Electric mobility shift assays (EMSAs) were carried out using cell extracts from HEK293 cells transiently transfected with the HA-Arix expression vector (Fig. 4B). Some cells were stimulated with forskolin to activate the PKA pathway and pretreated

with phosphatase inhibitors, okadaic acid or orthovanadate, prior to the preparation of the extracts. Using the HD3 probe, Arix-containing extract exhibited a supershift with an HA antibody. The formation of the supershift was robustly increased in the EMSA reaction with the Arix-containing extract prepared from forskolin treated cells. The supershift formation was absent in the mutant probe, HD3m, where the HD site is mutated, demonstrating that the supershift formation is dependent on the HD sites. Similar to our observation, others have also observed that the signal from the Arix-DNA-antibody complex is stronger than that from the Arix-DNA complex (170). Thus, supershift formation is often used as a way to identify Arix-containing specific DNA-protein complexes and the extent of supershift formation reflects Arix's DNA-binding activity. Stimulation of the PKA pathway consistently results in a substantial increase in the DNA-binding activity of Arix, from 2.5 to 3.5 fold between experiments, as quantitated by the intensity of the supershift.

Since Arix undergoes dephosphorylation upon PKA stimulation, as shown by *in vivo* ^{32}P labeling experiments (Fig. 2C), we asked whether phosphatase inhibitors can reverse the effect of PKA activation on the DNA-binding activity of Arix. Arix transfected cells were pretreated with okadaic acid or orthovanadate as serine/threonine and tyrosine phosphatase inhibitors, respectively, followed by stimulation of PKA activity by forskolin. In these phosphatase inhibitor treated cells, the formation of supershift significantly decreased, demonstrating weakened DNA-binding activity of Arix (Fig. 4B). The extent of the supershift formation was reversed close to the basal state of Arix.

We also examined the effect of PKA stimulation on the DNA-binding activity of Arix using the DB1 probe, which contains two HD sites. Similar to the results from EMSA reactions using HD3 probe, the Arix-containing extract from forskolin treated cells demonstrated a robust supershift formation, which was almost absent in forskolin-untreated cells (Fig. 4B). The supershift was no longer present in the mutant probe, HD2m, confirming the specificity of the supershift formation through the HD sites. When the intensity of super shift was quantitated, a 2 to 6 fold increase in supershift formation was observed between experiments. The phosphatase inhibitor treatment using okadaic acid as well as orthovanadate exhibited a reduction in the supershift formation, consistent to the EMSA reactions using the HD3 probe. The result that Arix's DNA-binding activity was blocked by phosphatase inhibitor treatment suggests that Arix gains DNA-binding activity by dephosphorylation upon PKA activation. Changes in the intensity of supershift formation was not due to the different content of Arix in cell extracts as indicated by the western blot (Fig. 4C), suggesting that post-translational modification of Arix by the PKA pathway alters the DNA-binding activity.

The results from EMSA analyses imply that Arix's DNA-binding activity is enhanced by a dephosphorylation event resulting from stimulation of the PKA pathway. Therefore, we also asked whether the phosphatase inhibitors affect transcription from the DBH promoter-reporter construct, DBH-Luc, in HepG2 cells. Treatment of orthovanadate prior to the forskolin stimulation reduced the total DBH promoter activity mediated by Arix and PKA activation by 40%, suggesting that inactivation of a dephosphorylation event, likely on Arix, prevents augmentation of DBH transcription (Fig. 5). When PKA-responsiveness was evaluated in the presence of Arix,

orthovanadate decreased the elevation in reporter gene activity from 3 fold to 1.4 fold. These results further suggest that dephosphorylation of Arix is involved in the PKA-responsiveness of DBH transcription. Collectively from the results described above, we hypothesize that constitutively phosphorylated Arix at the basal level undergoes dephosphorylation in a PKA-dependent manner, gaining DNA-binding activity and becoming transcriptionally active. The appropriate post-translational modification of Arix through phosphorylation/dephosphorylation may regulate the activation of the DBH gene transcription stimulated by PKA.

Phosphorylation sites resided in either or both the homeodomain and the N-terminus of Arix may participate in regulation of the DBH gene transcription. To further understand the mechanism involved in the PKA-regulated activity of Arix, we sought to define the phosphorylation sites on Arix responsible for the functional changes in DNA binding. Analyses of the primary amino acid sequence of Arix for consensus phosphorylation sites revealed that multiple phosphorylation sites are present in the C-terminus as well as in the homeodomain, but none in the N-terminus (Fig. 6A). These sites are potential targets for tyrosine kinase, CKII, PKC, and mitogen-activated protein (MAP) kinase. No PKA phosphorylation sites were predicted, consistent with the results presented in Fig. 2A and B. In order to map the phosphorylation sites of Arix, we first performed *in vitro* kinase assays using truncated Arix constructs (Fig. 6A) as substrates and PC12 cell nuclear extracts as a source of kinases. Phosphatase sensitive mobility shifts, indicative of phosphorylated form of proteins, were observed from ArΔN and HDAr constructs, consistent with the prediction that phosphorylation sites reside in the

homeodomain (Fig. 6B). However, the ArΔC construct did not exhibit phosphatase sensitive mobility shifts, even though the *in vitro* kinase assay with the homeodomain alone resulted in mobility shifts. It is possible that C-terminus truncation caused a structural change that masked the phosphorylation sites; therefore, phosphatase sensitive shifts were undetectable in this *in vitro* kinase assay system.

Previous experiments have demonstrated that truncation of the C-terminus does not reduce the transcriptional synergism between PKA and Arix on the DBH promoter activity (1). Therefore, we examined the phosphorylation state of the ArΔC construct by *in vivo* ^{32}P labeling. *In vitro* phosphorylation of the ArΔC construct was apparent (Fig. 6C) although ^{32}P incorporation was reduced, by 40-90% between two experiments, compared to full-length Arix (Table 1). The reduction in ^{32}P incorporation of the ArΔC construct can be attributed to the fact that multiple phosphorylation sites are present in the C-terminal segment while fewer phosphorylation sites are located in the homeodomain and/or the N-terminus of Arix. We also tested ^{32}P incorporation into the mutant ArΔC constructs, in which the predicted phosphorylation sites, tyrosine (Y25) or threonine (T30), of the homeodomain are substituted with phenylalanine and alanine, respectively. Mutation at the Y25 residue resulted in a further decrease in ^{32}P incorporation in comparison to the wild-type ArΔC (Fig. 6C). A 75-95 % reduction in ^{32}P incorporation was observed among two experiments (Table 1). In contrast, ^{32}P incorporation of the T30A mutant construct was comparable to ArΔC. These results imply that Y25, but not T30, is a likely target of phosphorylation *in vivo*.

To ascertain which amino acids are phosphorylated, ^{32}P-labeled Arix proteins were extracted from the gel shown in Fig. 6C and subjected to a phosphoamino acid

mapping. In this analysis, phospho-serine was evident, from both the full-length and C-terminus truncated Arix proteins, (Fig6. D). This result suggests that not only are there sites for serine kinases in the C-terminus, as predicted by consensus sequence analyses, but there are additional phosphorylation sites on serine in the homeodomain and/or the N-terminus, not predicted by the sequence analyses. However, we were not able to observe detectable signals for either phospho-tyrosine or phospho-threonine using either full-length Arix or ΔCAr proteins. Taken together, we conclude that there are multiple phosphorylation sites in Arix, residing the C-terminal, the homeo, and the N-terminal domains.

DISCUSSION

Arix has been suggested to serve as an integrator that coordinates multiple extracellular signals to mediate the expression of characteristic terminal differentiation genes for noradrenergic neurons in developing nervous systems (88) (148). The present study gives insight into the molecular mechanism underlying the transcriptional activity of Arix in coordination with the cAMP/PKA signaling pathway in the activation of DBH transcription. We demonstrate that Arix is constitutively phosphorylated at multiple amino acid residues in cell cultures. Stimulation of the PKA pathway leads to a significant decrease in the phosphorylation state of Arix, which coincides with greatly increased DNA-binding activity of Arix to the multiple HD sites within the DBH proximal promoter. The increase in Arix's DNA-binding activity is abolished by phosphatase inhibitor treatments. Consistently, the phosphatase treatment partly reduces the PKA-stimulated DBH promoter activity mediated by Arix. Taken together, the present findings imply that the transcriptional activity of Arix is negatively regulated by phosphorylation and that activation of the PKA pathway causes dephosphorylation of Arix, converting it to the transcriptionally competent form, and potentiating DBH gene transcription. Since the closely related homeodomain transcription factor, NBPhox/Phox2b, is also synergistic with PKA in the activation of DBH gene transcription (1), it is likely that the functional characteristics of Arix described in the present study are also shared with NBPhox.

Possible kinases and phosphatases that regulate phosphorylation of Arix Our previous studies showed that transcription from the rat DBH promoter is significantly enhanced together with Arix and activation of the PKA pathway (150). PKA-dependent recruitment of AP1 proteins, Fos and Jun, to the CRE/AP1 site is a part of the mechanism of PKA-stimulated DBH transcription (149). We also identified the transcriptional coactivator, CBP, as a signal-dependent facilitator of PKA-stimulated DBH transcription (148). The present results further extend the molecular mechanism of DBH gene transcription by demonstrating that the post-translational modification of Arix is regulated by PKA, and influencing its DNA-binding activity. Consistent with the fact that phosphorylation of Arix is constitutive under basal condition, PKA does not directly phosphorylate Arix. The kinase responsible for phosphorylation of Arix remains to be determined. One such kinase would be MAP kinase, which is activated by a variety of growth factors as well as neurotrophins. Leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) are known to induce a neurotransmitter-phenotype switch in sympathetic neurons from noradrenergic to cholinergic during development (32). During the phenotype switch, LIF and CNTF cause downregulation of DBH gene transcription, which can be blocked by a MAP kinase inhibitor, PD98059 (S. Dziennis, J. Hofmann, E. Lewis, and B. Habecker, Abst. 31st annual meeting Society for Neuroscience, abstr.364.27, 2001). This observation suggests that activation of MAP kinase negatively regulate DBH transcription. Possibly, MAP kinase phosphorylates Arix, thereby reduces its transcriptional activity. It is plausible that the growth factors present in a cell's local environment activate MAP kinase, which, in turn, phosphorylates Arix. The level of phosphorylation of Arix may influence basal transcription of the DBH gene. When cells

encounter another environmental cue that activates the PKA pathway, phosphorylated Arix is converted to a dephosphorylated form, gaining stronger transcriptional activity and enhancing the DBH transcription.

It is of interest to identify the phosphatase that dephosphorylates Arix in response to PKA stimulation. In fact, increasing lines of evidence suggest that protein phosphatase plays an important role in developmental processes (61, 147). Among a diverse family of protein phosphatases, protein phosphatase 2A (PP2A), a serine/threonine phosphatase, is an attractive candidate to investigate whether it alters the phosphorylation state of Arix. The regulatory B subunit of PP2A determines the substrate specificity of the enzyme. In particular, B56δ is highly expressed in brain and concentrated in the nucleus as a phosphoprotein *in vivo* (97). Furthermore, *in vitro* studies suggest that phosphorylation of PP2A by PKA positively regulates its phosphatase activity (158).

Dephosphorylation of Arix positively regulates its transcriptional potential. As demonstrated by EMSA (Fig 3), Arix's DNA-binding activity is greatly enhanced by stimulation of the PKA pathway, which appears to lead to dephosphorylation of Arix. In addition, serine/threonine as well as tyrosine phosphatase inhibitor treatments abrogated the enhanced DNA binding of Arix, suggesting that phosphorylation at both serine/threonine and tyrosine residues play an inhibitory role in DNA binding. *In vitro* studies suggest that the homeodomain can be phosphorylated. In principle, phosphorylation in the DNA-binding domain introduces a negative charge, which is unfavorable for DNA binding since the backbone of DNA is negatively charged in character. If phosphorylation occurs within or nearby the homeodomain of Arix, removal

of phosphorylation would be an obligatory step to confer DNA-binding activity of Arix. With regard to the homeodomain proteins, Berry and Gehring reported that the *Drosophila* Hox protein, sex combs reduced (SRC), which determines the identity of specific segments, is regulated by phosphorylation (9). *In vivo* analyses using transgenic flies revealed that a mutant form of SRC mimicking constitutive phosphorylation in the N-terminal arm of the homeodomain is functionally inactive. Dephosphorylation is apparently necessary to switch SRC in an active state, possibly by PP2A, since a yeast two-hybrid screen revealed the interaction between SRC and PP2A .

The functional importance of the phosphorylation state of Arix is also suggested by *in vitro* protein-protein interaction assays (Fig. 3). Homo- and heteromerization of Arix and NBPhox *in vitro* occurs preferably in the dephosphorylated forms of these proteins, rather than the phosphorylated forms. Whether binding of Arix to DNA requires protein dimerization is unknown at present. If this is the case, phosphorylation at the dimerization interface may decrease dimerization, thereby inhibiting DNA binding. Alternatively, since the DBH proximal promoter carries multiple homeodomain recognition sites, multimerization of Arix and NBPhox may confer cooperativity in DNA binding. Upon stimulation of the PKA pathway, dephosphorylated Arix may undergo multimer formation, stabilizing the transcriptional machinery at the promoter, thereby enhancing DBH transcription.

Another functional aspect of phosphorylation of a transcription factor is the regulation in cellular localization (57, 163). An example of a protein which is active in its dephosphorylated state is NF-AT. When calcineurin dephosphorylates NF-AT in response to a rise in intracellular calcium levels, nuclear import is induced (30). In the

case of Arix, the putative nuclear localization signal is located in the N-terminal arm of the homeodomain. However, involvement of Arix phosphorylation in cellular targeting is dubious since we have observed Arix only in the nucleus under basal conditions (1).

Functional phosphorylation sites, possibly, locate to either the homeodomain or the N-terminus of Arix. Our results (Fig 6) suggest that target amino acid residues for phosphorylation appear to locate to either the homeodomain or the N-terminus of Arix. Previously, we have identified the transactivation domain of Arix in the N-terminus (aa1-83). In addition, ArΔC can activate the DBH promoter comparable to the full-length Arix under basal and PKA-stimulated conditions (1). Therefore, it is conceivable that phosphorylation in the N-terminus and/or the homeodomain functionally modulates the transcriptional potential of Arix by influencing the DNA-binding activity. The phosphoamino acid mapping analyses (Fig. 6) revealed the presence of phosphorylation at serine residues in either the homeodomain or N-terminus of Arix. We have also observed 20% reduction in the DBH promoter activity by Arix and PKA upon okadaic acid treatment to block serine/threonine phosphatases (Adachi and Lewis unpublished data). Thus, dephosphorylation of serine may be a part of the mechanism to regulate the transcriptional activity of Arix.

The results from EMSA (Fig. 4) and DBH reporter assays (Fig. 5) using orthovanadate, a tyrosine phosphatase inhibitor, strongly suggest that tyrosine phosphorylation of Arix negatively regulates Arix's DNA-binding as well as DBH promoter activity. Although the results with the Y25F mutant suggest that this residue is phosphorylated, it is, however, unclear whether the Y25 residue of the homeodomain is

the target phosphorylation sites since the results from the phosphoamino acid mapping analyses demonstrated the presence of only phosphoserine (Fig. 6). Further analyses are necessary to confirm tyrosine phosphorylation at the Y25 residue. Nevertheless it is worthwhile to note that the Y25 residue is absolutely conserved among pair and paired-like homeodomain classes (Fig. 7A). Furthermore, the predicted structure of Arix revealed that the hydroxyl group of the Y25 residue makes a direct contact with the phosphate backbone of DNA through a hydrogen bond and that modification by the negatively charged phosphorylation at the Y25 becomes repulsive and too bulky to make a contact with the backbone of DNA (Adachi and Lewis unpublished data). Therefore, the Y25 residue is an attractive candidate for the functional phosphorylation site. If this is true, conservation of the Y25 residue suggests that regulated phosphorylation of the Y25 residue is a common mechanism to govern the functional activity among the paired and paired-like homeodomain proteins. We have tested whether the mutant Arix construct, in which the Y25 residure is replaced with a non-phosphorylatable residue, phenylalanine (ArY25F), can act as a constitutitvely active Arix on DBH transcription. This mutant construct, however, was unable to bind to DNA as tested by EMSA, and therefore resulted in a decrease in PKA-stimulated DBH transcription (M. Adachi and E. J. Lewis unpublished data). It is likely that the ArY25F failed to contact DNA due to the absence of the hydroxyl group in the phenylalanine residue.

Arix may integrate extracellular signals through the cAMP/PKA pathway and enhance DBH gene transcription during noradrenergic differentiation. During embryogenesis, noradrenergic differentiation is believed to be triggered by BMPs,

secreted proteins of the transforming growth factor ß (TGFß) superfamily. Exposure to BMPs leads to the sequential induction of Mash1 and Phox2a/Arix expression (88, 138). However, induction of TH and DBH expression in mammalian neuronal crest stem cell culture requires the activation of PKA, through the elevation of intracellular cAMP (88). In other developmental processes such as renal branching morphogenesis (48) and chondrogenesis (80), BMP2 has been demonstrated to increase PKA activity. Therefore, it is plausible that BMP2/4, which have been shown to induce the expression of TH and DBH *in vivo* (88, 123), can stimulate the cAMP/PKA pathway in noradrenergic precursor cells.

Based on our results, the model for the regulation of DBH gene transcription is outlined in Fig. 7B. Arix, the noradrenergic tissue specific transcription factor, is present in a constitutively phosphorylated form under the basal condition, which is a transcriptionally reduced state. Phosphorylated Arix has weak DNA-binding activity that contributes to the minimal activation of DBH transcription. During development, cells encounter an environmental cue that activates the cAMP/PKA pathway, which in turn stimulates protein phosphatases by an unknown mechanism. Phosphatases will, then, dephosphorylate Arix at either or both the homeodomain or the N-terminus, locking in an active state by gaining a strong DNA-binding activity. As a result, dephosphorylated Arix becomes competent to fully activate DBH transcription. Thus, the present study highlights that the phosphorylation state of Arix shifts its functional activity through DNA binding and that Arix may serve as a signal-dependent tissue-specific transcription factor that consolidates an extracellular cue, promoting a noradrenergic fate. The results

of our present study provide the missing link between Arix and activation of the cAMP/PKA pathway.

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Table 3.1 ^{32}P incorporation (%)

construct	Experiment 1	Experiment 2
Arix	100	100
ArΔC	12.4	62.4
ArΔC Y25F	0.5	15.1
ArΔC T30A	22.0	65.1

^{32}P incorporation was quantitatively analyzed using a Phosphoimager and expressed as a percentage of thte full-length Arix phosphorylation.

Figure 3.1. Fos and Jun are not sufficient to fully activate DBH promoter by Arix under the PKA-stimulated condition. DBH promoter activity was examined in HepG2 cells where DBH-Luc (750ng), HA-Arix (100ng) and pRL-null (100ng) constructs were transiently transfected either with or without the expression plasmids of Fos and Jun (200ng each). The next day, cells were treated with 20μM of forsklin (fsk) for 7hr and harvested for dual luciferase assays. Values for firefly luciferase activity were normalized to those for the renilla luciferase activity and calculated relative to the basal promoter activity in the absence of Arix and forskolin. Thus, the mean values of fold luciferase activity for the basal control is equal to 1. Experiments were done in triplicate and repeated three times with similar results. Since the magnitude of responses were variable in each experiment, the data from one representative experiment is shown and bars present mean + SD.

Figure 3.1

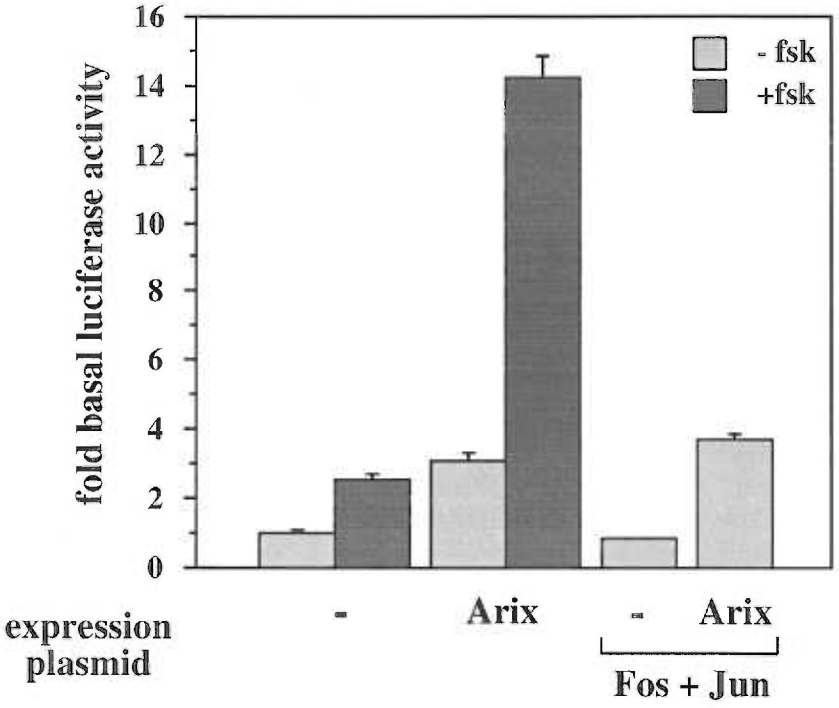
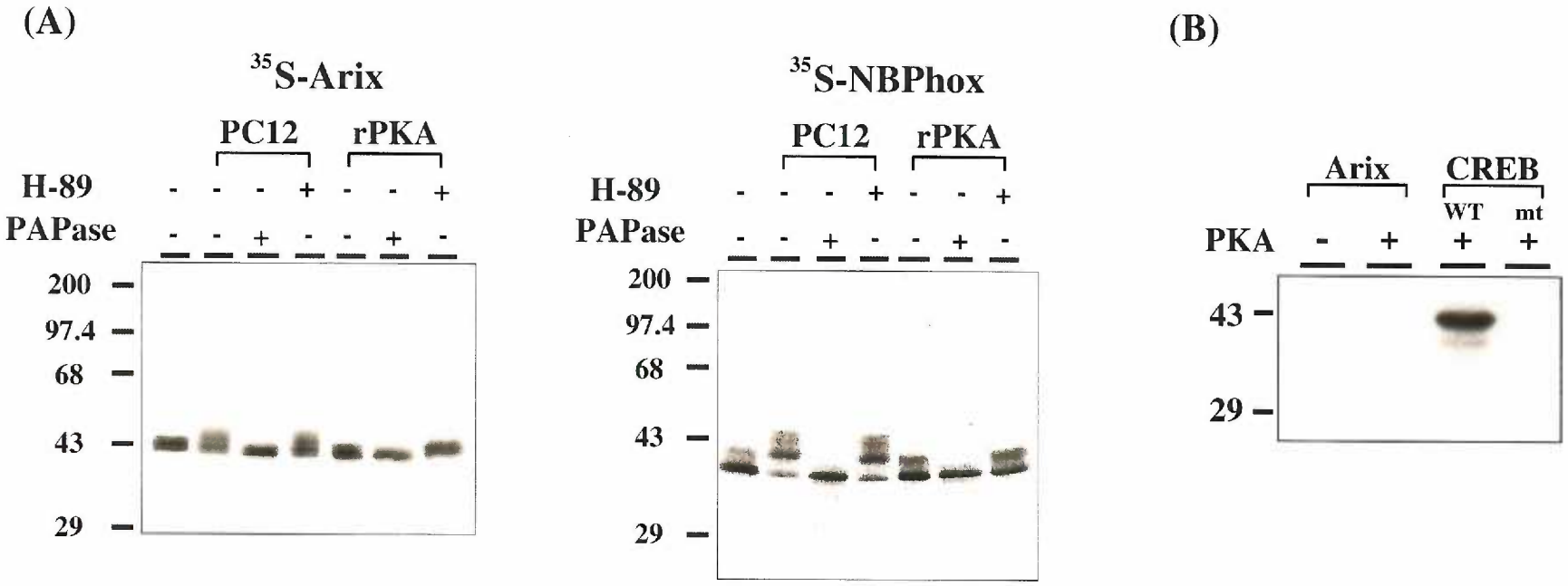

16
14
12
10
8
6
4
2
0
fold basal luciferase activity
- fsk
+fsk
-
Arix
-
Arix
Fos + Jun
expression plasmid

Figure 3.2. Arix is phosphorylated both *in vitro* and *in vivo*. (A) ^{35}S-labeled IVT Arix (left panel) or NBPhox (right panel) was subjected to the *in vitro* kinase assay using PC12 nuclear extracts or recombinant PKA (rPKA) as a source of kinases. Some of assays included 5μM of H-89, a PKA specific inhibitor. The reaction products were resolved on 10% SDS-PAGE followed by autoradiography. Potato acid phosphatase (PAPase) was added to dephosphorylate the phosphorylated products; thereby, PAPase sensitive mobility shifts are evidence of phosphorylation. **(B)** Bacterially produced Arix, wild-type CREB (WT), and mutant CREB (mt) were incubated in the kinase reaction buffer with rPKA in the presence of [γ-^{32}P]ATP. Reaction products were resolved on 10% SDS-PAGE, followed by autoradiography. The amounts of Arix and CREB loaded in each lane are approximately eaqual as examined by coomassie-blue staining of a gel (data not shown). **(C)** HEK293 cells were transfected with DBH-Luc (750ng), HA-Arix (100ng), and pRL-null (100ng) constructs either with or without the expression vector of PKA catalytic subunit (250ng) to examine the DBH promoter activity. HA6.1 is a backbone of the Arix expression vector. Similar to HepG2 cells, DBH promoter was synergistically activated together with Arix and PKA in HEK293 cells. **(D)** HEK293 cells were transiently transfected with 5μg of HA-Arix plasmind either with or without 2.5 μg of the PKA expression plasmid. Next day, cells were metabolically labeled with ^{32}P-orthophosphate for 4 hrs and harvested to prepare cell lysates. HA-Arix was immuoprecipitated with an HA antibody and resolved on 10% SDS-PAGE, followed by autoradiography (middle panel as indicated by ^{32}P). Immunoprecipitated Arix was also subjected to western blot and stained with HA antibody (bottom panel as indicated by αHA) to examine the amount of Arix. ^{32}P incorporation was quantitatively analyzed

using a Phosphoimager. Changes in ^{32}P incorporation are expressed as a percentage of Arix phosphorylation under the basal condition (top panel). Bars present mean values + range from two independent experiments.

Figure 3.2


(A)
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rPKA
H-89
PAPase
200
97.4
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rPKA
H-89
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(B)
Arix
CREB
WT
mt
PKA
43
29

Figure 3.2

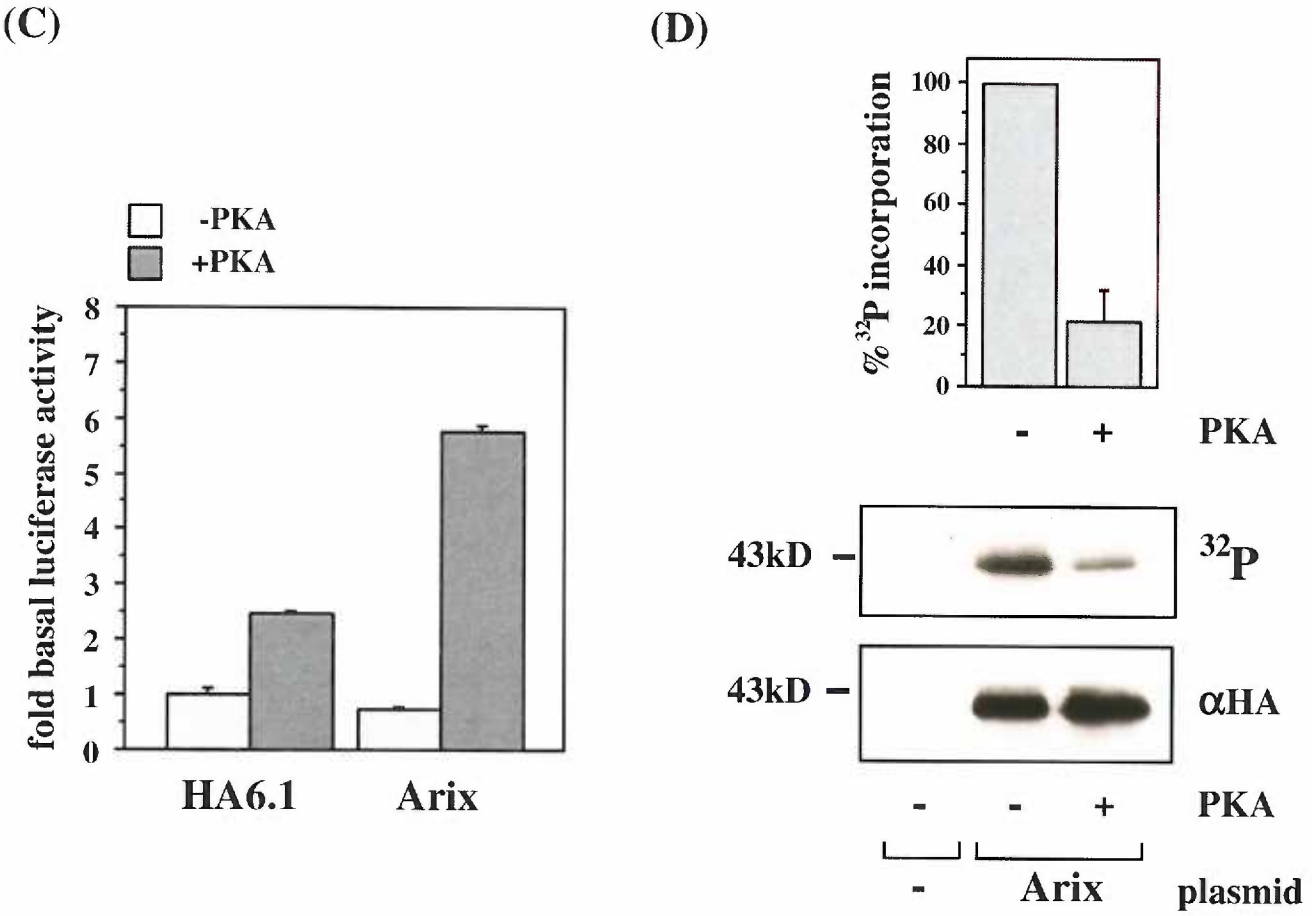

(C)
-PKA
+PKA
fold basal luciferase activity
0
1
2
3
4
5
6
7
8
HA6.1
Arix
(D)
% 32P incorporation
0
20
40
60
80
100
-
+
PKA
43kD
32P
43kD
αHA
-
-
+
PKA
-
Arix
plasmid

Figure 3.3. Dephosphorylated forms of Arix and NBPhox interact preferably *in vitro*. **(A)** ^{35}S-IVT Arix or NBPhox was incubated with PC12 cell nuclear extracts as a source of kinases. The reaction products were resolved on SDS-PAGE followed by autoradiograph. PAPase sensitive mobility shifts were observed as an evidence of phosphorylation. **(B)** *In vitro* protein-protein interaction assays were carried out in combination with kinase assays to determine whether phosphorylated or dephosphorylated forms of Arix and NBPhox preferably undergo protein-protein interaction. Prior to protein-protein interaction assays, ^{35}S-IVT proteins were treated with PC12 nuclear extracts in kinase buffer as described in Methods and Materials. All reaction products in the kinase assay were then incubated with TALON metal resin in the presence of recombinant His-Arix or His-NBPhox. His-Arix (right panel) or His NBPhox (left panel) interacting proteins were analyzed by SDS-PAGE and detected by ^{35}S signals. "Sup" indicates unbound fractions collected from the supernatant of the binding reaction before washing TALON metal affinity resin, showing that phosphorylated ^{35}S-IVT products were abundant in the unbound fraction. **(C)** After the above *in vitro* binding assays, washed resin was further treated with micrococcal nuclease prior to SDS-PAGE analyses to control for the possibility that these protein-protein interactions are non-specifically mediated by association with DNA. The binding assays with and without micrococcal nuclease treatment were carried out in parallel and examined by autoradiography with an exactly same exposure time.

Figure 3.3

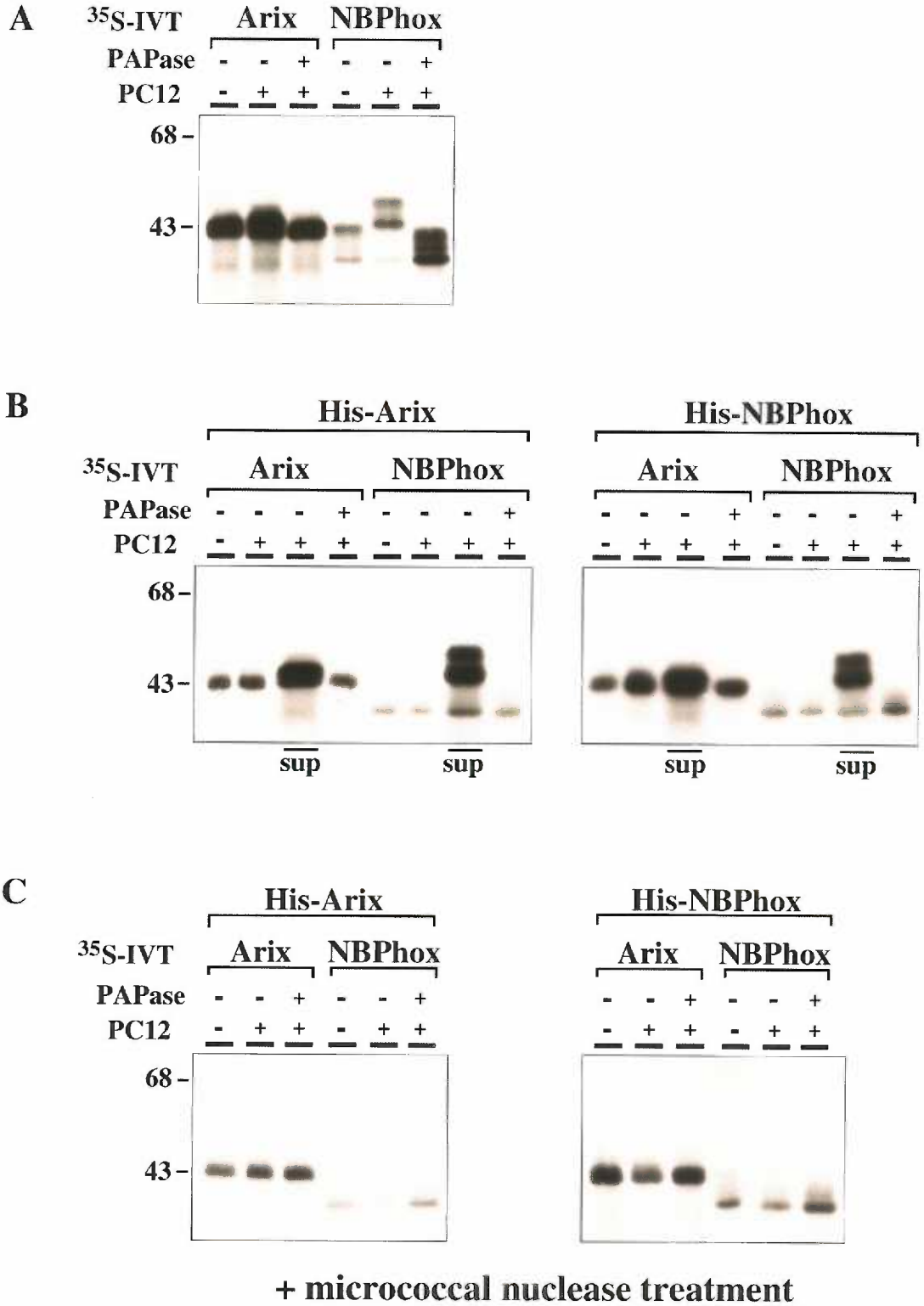
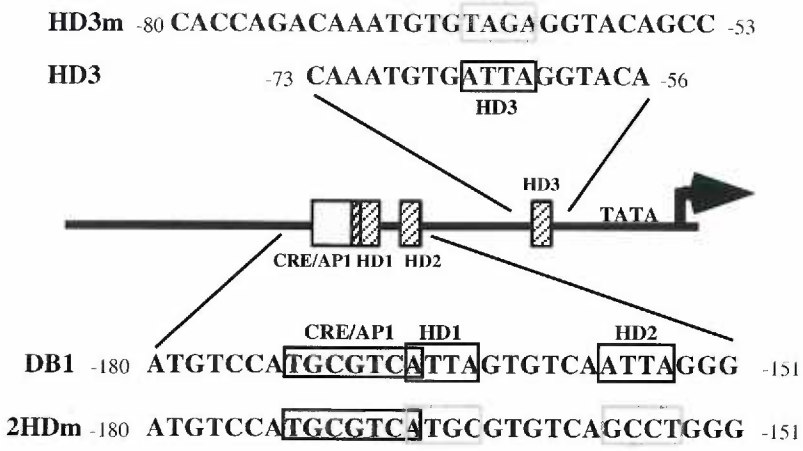

A
35S-IVT
Arix
NBPhox
PAPase
PC12
68
43
B
His-Arix
His-NBPhox
sup
C
+ micrococcal nuclease treatment

Figure 3.4. The DNA-binding activity of Arix was greatly enhanced under the PKA-stimulated condition and reversed by phosphatase inhibitor treatments. (A) A schematic diagram of the DBH promoter is depicted with regulatory elements, CRE/AP1 site and homeodomain recognition sites, HD1, 2, and 3. Wild type and mutant oligonucleotides used in the following EMSA are presented in detail with sequences. **(B)** Using HEK293 cell extracts, EMSA was carried out with wild type probes, HD3 and DB1, as well as mutant probes, 2HDm and HD3m, which lack the HD sites. To make cell extracts, HEK293 cell were transiently transfected with 5μg of HA-Arix. One day after transfection, cells were stimulated with fsk for 1hr and harvested for the preparation of cell extracts. Some cells were treated with phosphatase (PPase) inhibitors, 50nM of okadaic acid (OA) or 1mM of orthovanadate (VO), for 30min prior to the fsk stimulation. As a competition assay of EMSA, 50ng of an unlabeled oligonucleotide was added in the binding reaction (cold). To evaluate the DNA binding activity of Arix, 0.5μg of HA antibody was co-incubated (αHA). The formation of a supershift complex was observed from wild type probes, HD3 and DB1, as indicated by an open arrowhead. The super shift complexes created by HD3 and DB1 probes were quantitated by using a Phosphoimager. The intensity of a super shift under the basal condition, where only Arix is included in cell extracts with no treatments of fsk, OA, and VO, is calculated as 1. Thus, the fold intensity is relative to the intensity of the basal state. The mobility shifts indicated by a closed arrow in DB1 and 2HDm probes are non-specific DNA-protein complexes. **(C)** The amounts of Arix in HEK293 cell extracts used in the above EMSA were examined by a western blot. 2.5μg of cell extracts were separated on a 10% SDS-PAGE and HA-tagged Arix was stained with the HA antibody.

Figure 3.4

(A)


HD3m -80 CACCAGACAAATGTGTAGAGGTACAGCC -53
HD3 -73 CAAATGTGATTAGGTACA -56
HD3
HD3
TATA
CRE/AP1 HD1 HD2
CRE/AP1 HD1 HD2
DB1 -180 ATGTCCATGCGTCATTAGTGTCAATTAGGG -151
2HDm -180 ATGTCCATGCGTCATGCGTGTCAGCCTGGG -151

(C)

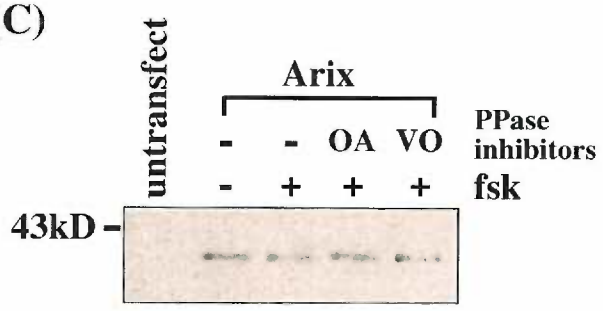

untransfect
Arix
- - OA VO
PPase inhibitors
- + + + fsk
43kD -

Figure 3.4

(B)

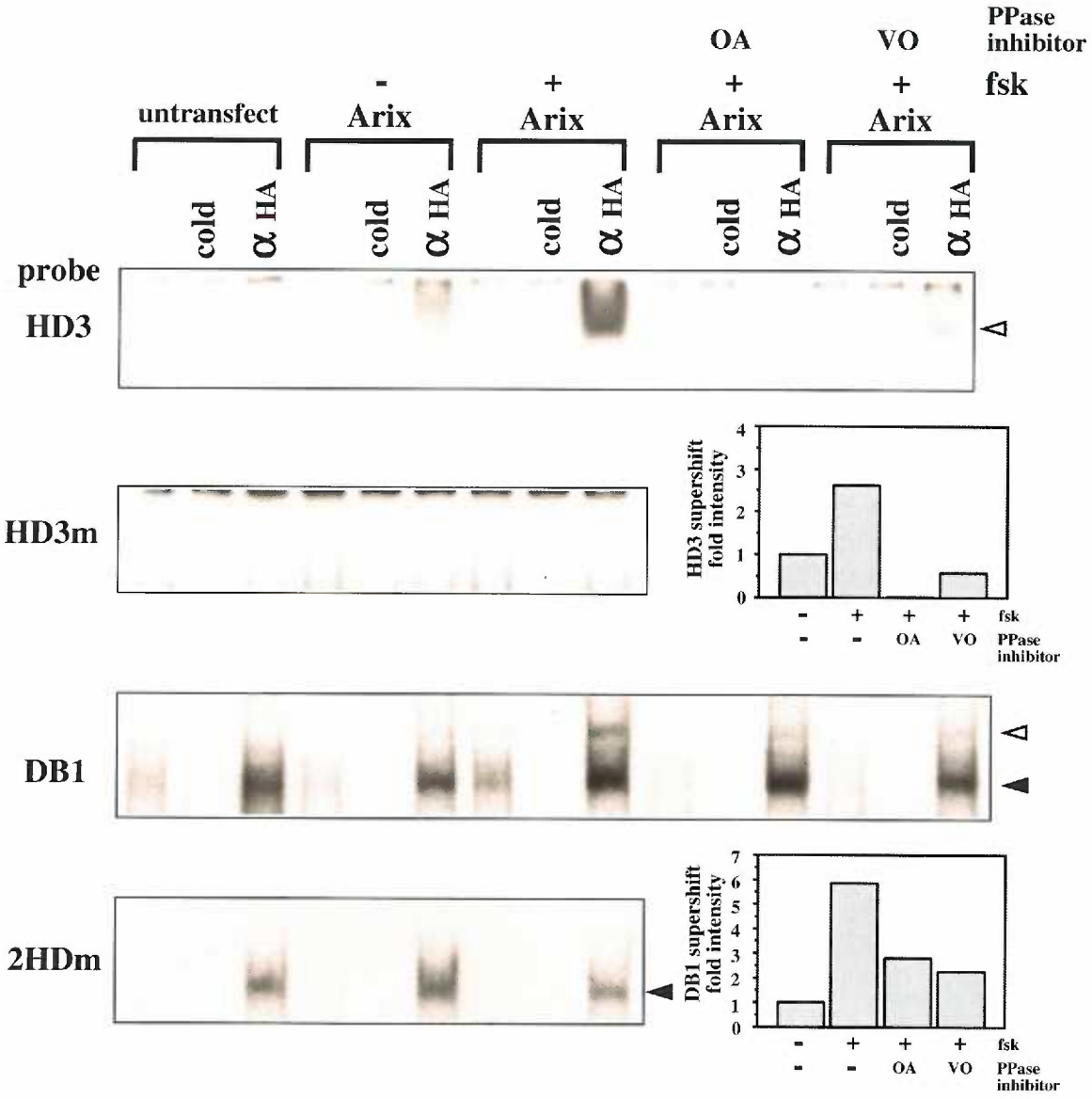

PPase inhibitor
OA
VO
fsk
untransfect
- Arix
+ Arix
+ Arix
+ Arix
cold
α HA
probe
HD3
HD3m
HD3 supershift fold intensity
DB1
2HDm
DB1 supershift fold intensity
PPase inhibitor

Figure 3.5. A tyrosine phosphatase inhibitor significantly reduces the PKA responsiveness mediated by Arix, leading to decrease in the overall DBH transcription. DBH promoter activity was examined in HepG2 cells, which were transfected with 750ng of DBH-Luc, 100ng of HA-Arix, and 100ng of pRL-null plasmid constructs. One day after transfection, cells were stimulated with 20μM of fsk for 7hrs and harvested for luciferase assays. Some of them were pre-treated with 1mM of orthovanadate (VO) prior to the fsk stimulation. Values for firefly luciferase activity were normalized to those for the renilla luciferase activity and caluculated relative to the basal promoter activity in the absence of Arix and forskolin. Thus, the mean values of fold luciferase activity for the basal control is equal to 1. Values present mean + SD from one representative experiment. Experiments were repeated three times with similar results. PKA-responsiveness was expressed by a ratio of fold luciferase activity under the basal condition to that under the fsk-stimulated condition.

Figure 3.5

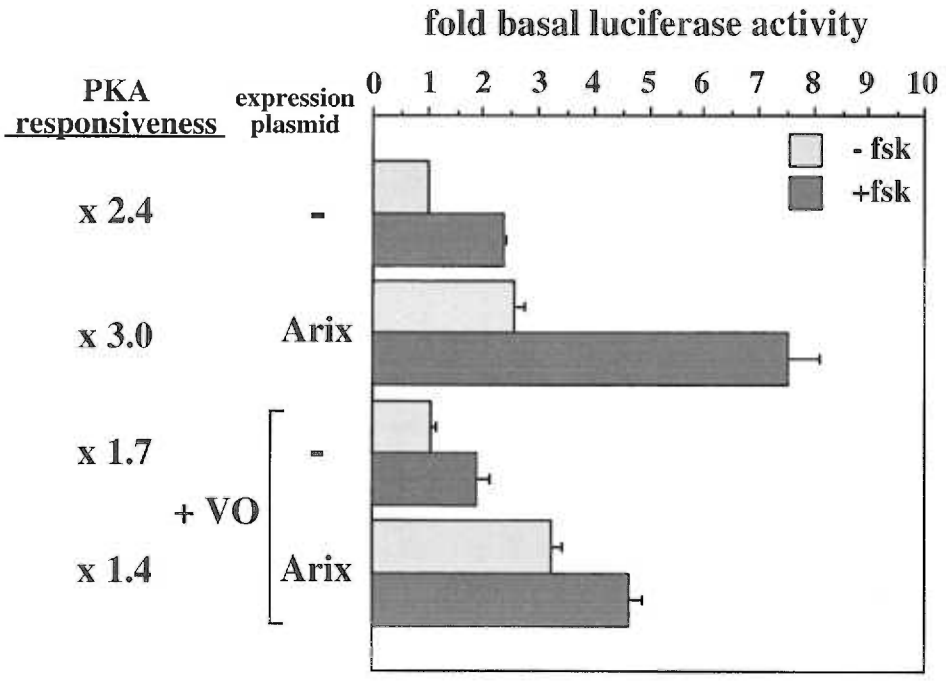

fold basal luciferase activity
PKA responsiveness
expression plasmid
0 1 2 3 4 5 6 7 8 9 10
- fsk
+fsk
x 2.4
-
x 3.0
Arix
x 1.7
-
+ VO
x 1.4
Arix

Figure 3.6. Functional phosphorylation sites may reside in the homeodomain and/or the N-terminus of Arix. **(A)** Schematic diagram of full-length and C-terminus truncated Arix constructs either with or without a single amino acid change in the homeodomain used in the following experiment is depicted. The diagram also includes consensus phosphorylation sites, which were predicted by using GeneWorks program (Intelli Genetics). For the prediction of MAP kinase phosphorylation sites, a mininal concensus sequence, S/T-P, was used (Davis et al., 1993). All constructs were tagged with HA as indicated by a black box. **(B)** Truncated Arix constructs were *in vitro* translated with ^{35}S and subjected to the *in vitro* kinase assay using PC12 nuclear extracts as a source of kinases. The reaction products were resolved on 10% SDS-PAGE followed by autoradiograph. Potato acid phosphatase (PAPase) was added to dephosphorylate the phosphorylated products; thereby, PAPase sensitive mobility shifts are evidence of phosphorylation. **(C)** HEK293 cells were transiently transfected with one of the Arix constructs (5μg) shown in (A). Next day, cells were metabolically labeled with ^{32}P-orthophosphate for 4 hrs and harvested to prepare cell lysates. Arix was immunoprecipitated with the HA-antibody and resolved on 10% SDS-PAGE, followed by autoradiography. ^{32}P signals incorporated into Arix and ArΔC proteins were indicated by a closed and open arrow, respectively. **(D)** ^{32}P labeled Arix and ArΔC proteins were exratced from the gel shown in (C) and subjected to two-dimensional phosphoamino acid mapping. The cross marks the origin of samples. Standards for phospho-serine, threonine, and tyrosine were visualized by ninhydrin reagent, circled, and indicated by S, T, and Y, respectively.

Figure 3.6

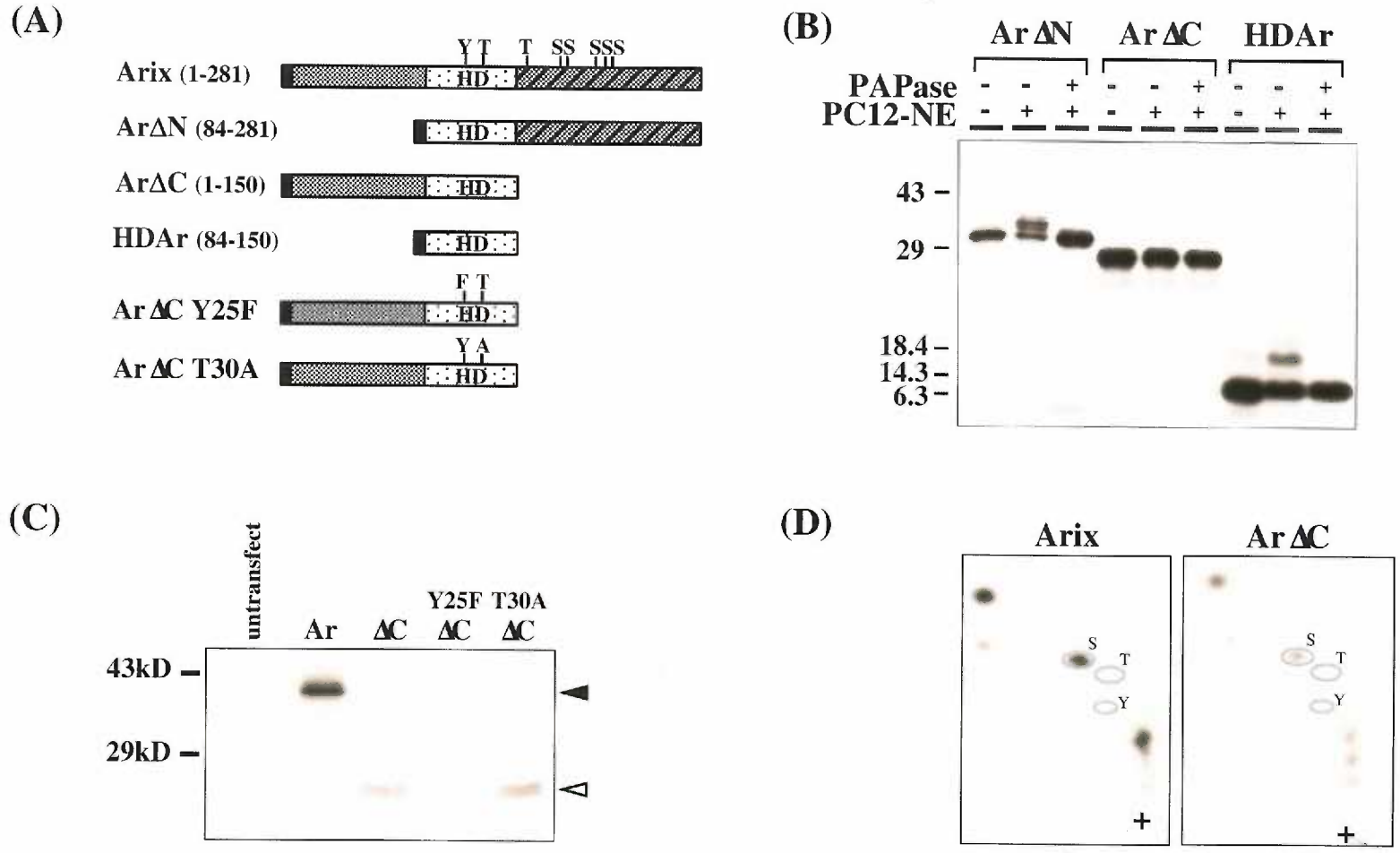

(A)
Arix (1-281)
ArΔN (84-281)
ArΔC (1-150)
HDAr (84-150)
Ar ΔC Y25F
Ar ΔC T30A
HD
Y T
T
SS
SSS
F T
Y A
(B)
Ar ΔN
Ar ΔC
HDAr
PAPase
PC12-NE
43 –
29 –
18.4 –
14.3 –
6.3 –
(C)
untransfect
Ar
ΔC
Y25F ΔC
T30A ΔC
43kD –
29kD –
(D)
Arix
Ar ΔC
S
T
Y
+

Figure 3.7. (A) Tyrosine 25 is conserved throughout the paird-like and paird classes of homeodomain proteins. The homeodomain consists of three helices; helix1, 2, and 3. Amino acid sequences within the homeodomain of paird-like and paird homeodomain proteins were aligned. Identical and similar amino acids are boxed and shaded, respectively. Y25 and T30 resides are marked with *. **(B)** A proposed model for the transcriptional machinery involved in PKA-mediated DBH transcription is depicted. When noradrenergic tissue specific transcription factors, Arix, is expressed in a cell, the DBH promoter is competent to drive basal transcription. In basal state, Arix is likely to be constitutively phosphorylated. However, its DNA binding activity is minimal. While the stimulation of the cAMP/PKA pathway leads to the recruitment of Fos/Jun complex at the CRE/AP1 site, resulting in a small activation of the DBH transcription. When two events, the expression of Arix and cAMP/PKA activation, occurs together, Arix becomes dephosphorylated by an unknown phosphatase. Dephosphorylated Arix gains stronger DNA binding activity and stabilizes the transcriptional machinery at the DBH promter, leading to enhancement of the DBH transcription.

Figure 3.7

A

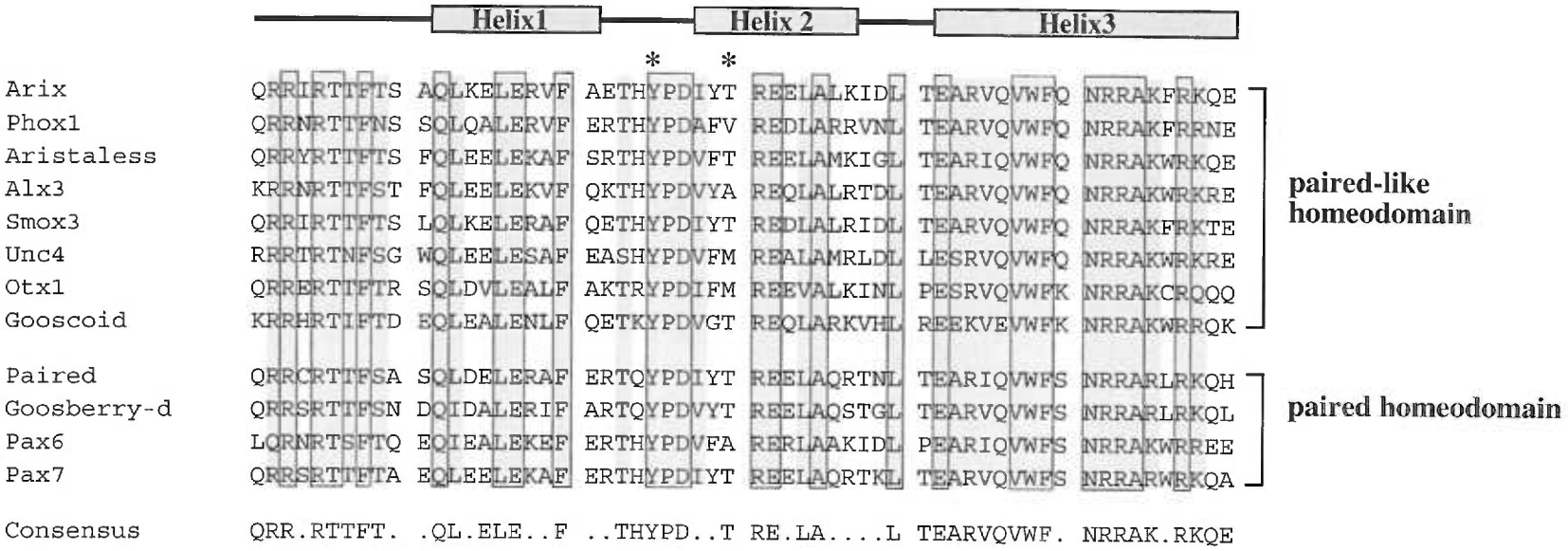
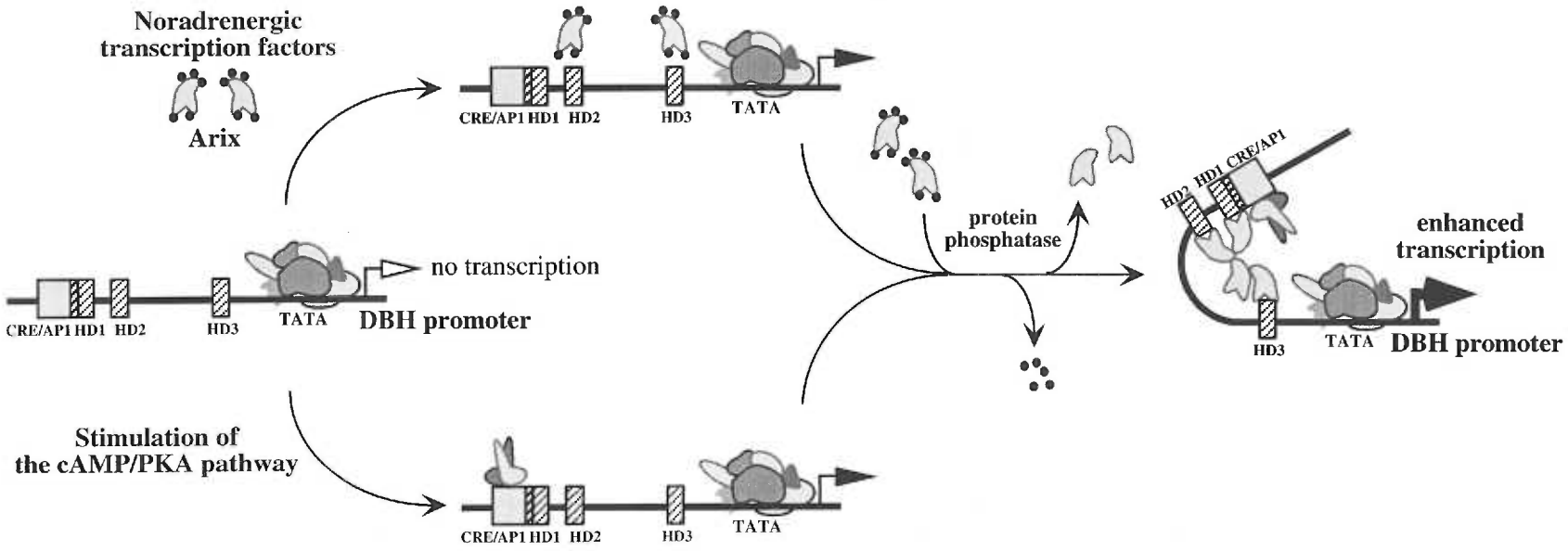

Helix1
Helix 2
Helix3
Arix QRRIRTTFTS AQLKELERVF AETHYPDIYT REELALKIDL TEARVQVWFQ NRRAKFRKQE
Phox1 QRRNRTTFNS SQLQALERVF ERTHYPDAFV REDLARRVNL TEARVQVWFQ NRRAKFRRNE
Aristaless QRRYRTTFTS FQLEELEKAF SRTHYPDVFT REELAMKIGL TEARIQVWFQ NRRAKWRKQE
Alx3 KRRNRTTFST FQLEELEKVF QKTHYPDVYA REQLALRTDL TEARVQVWFQ NRRAKWRKRE
Smox3 QRRIRTTFTS LQLKELERAF QETHYPDIYT REDLALRIDL TEARVQVWFQ NRRAKFRKTE
Unc4 RRRTRTNFSG WQLEELESAF EASHYPDVFM REALAMRLDL LESRVQVWFQ NRRAKWRKRE
Otx1 QRRERTTFTR SQLDVLEALF AKTRYPDIFM REEVALKINL PESRVQVWFK NRRAKCRQQQ
Gooscoid KRRHRTIFTD EQLEALENLF QETKYPDVGT REQLARKVHL REEKVEVWFK NRRAKWRRQK
Paired QRRCRTTFSA SQLDELERAF ERTQYPDIYT REELAQRTNL TEARIQVWFS NRRARLRKQH
Goosberry-d QRRSRTTFSN DQIDALERIF ARTQYPDVYT REELAQSTGL TEARVQVWFS NRRARLRKQL
Pax6 LQRNRTSFTQ EQIEALEKEF ERTHYPDVFA RERLAAKIDL PEARIQVWFS NRRAKWRREE
Pax7 QRRSRTTFTA EQLEELEKAF ERTHYPDIYT REELAQRTKL TEARVQVWFS NRRARWRKQA
Consensus QRR.RTTFT. .QL.ELE..F ..THYPD..T RE.LA....L TEARVQVWF. NRRAK.RKQE
paired-like homeodomain
paired homeodomain

Figure 3.7

B


Noradrenergic
transcription factors
Arix
CRE/AP1 HD1 HD2
HD3
TATA
protein
phosphatase
enhanced
transcription
HD2 HD1 CRE/AP1
HD3
TATA
DBH promoter
no transcription
CRE/AP1 HD1 HD2
HD3
TATA
DBH promoter
Stimulation of
the cAMP/PKA pathway
CRE/AP1 HD1 HD2
HD3
TATA

Chapter Four

Discussion and Future Directions

Summary

The overall goal of this thesis is to understand the molecular mechanism underlying activation of the DBH gene transcription through noradrenergic transcription factors, Arix/Phox2a and NBPhox/Phox2b. The first part of the thesis focused on the characterization of NBPhox in comparison to Arix in genetic, biochemical, and functional aspects. Arix and NBPhox are located on different chromosomes, yet, they share very similar gene structure. They exhibit indistinguishable and independent transcriptional regulatory properties on the DBH promoter. It is likely that both transcription factors activate the DBH gene in a similar mechanism, since the transactivation domain has been identified in the N-terminus of Arix, which bears 50% identity with NBPhox. These results suggest that the functional redundancy of the similar paired-like homeodomain proteins, Arix and NBPhox, have evolved to assure appropriate transcription of critical genes, as well as to allow subtle differences in the patterns of target gene expression.

In the second part of the thesis, I have initiated to elucidate the molecular mechanism of Arix underlying the activation of the DBH gene. In particular, it was of interest to examine whether post-translational modification of Arix influences the DBH gene transcription, since Arix's transcriptional activity is prominently potentiated by simultaneous stimulation of the PKA pathway. Activation of the PKA pathway possibly correlates with the *in vivo* situation during development. Environmental signals may activate second messenger pathways and act in concert with other molecules, such as transcription factors, in order to direct a neurogenic program for noradrenergic specification. With this respect, Arix is a reasonable candidate as a signal-integrator.

The major finding was that phosphorylation status of Arix is an important regulatory modification to switch Arix to a transcriptionally active form. Under basal conditions, Arix is constitutively phosphorylated, but becomes dephosphorylated upon activation of the PKA pathway. The dephosphorylation results in an increase in the DNA-binding activity of Arix and subsequent enhancement of the DBH gene transcription. Kinases and phosphatases that regulate the phosphorylation state of Arix remain unknown at present. Functional phosphorylation sites need to be determined as well. Nevertheless, evidence presented in the second part of this thesis support the idea that Arix can functionally integrate cellular signals through post-translational modification, controlling DBH gene transcription.

Brachyury-like motif

Since the N-terminus of Arix is necessary for the transcriptional activity, a Blast search was performed in order to seek a homologous region with putative transactivation domains of other transcription factors. A Blast search using N-terminal amino acids (1-89) of Arix revealed that a 15 amino-acid stretch (aa 61-75) exhibited high homology with a member of T-box gene family, Brachyury, and a paired-box protein, Pax9 (Fig. 4.1). This short stretch of homology was given the name the Brachyury-like motif to reflect the abundance of homologues with this motif. Both Brachyury and Pax9 are putative transcription factors that play important roles in early development. Brachyury is required for the formation of posterior mesoderm and for the axial development (144). Pax9 plays an essential role during the development of organs derived from endoderm, mesoderm, and neural crest (118). Functional domain studies of the Brachyury protein of mouse and Xenopus and Pax9 from zebrafish, indicate that the Brachyury-like motif is located between the transactivation and the DNA-binding domains in each case (17, 73, 112). Although this motif within Brachyury or Pax9 does not appear to be directly involved in either transcriptional activation or DNA binding, it is conserved among a variety of different species, suggesting that this short peptide stretch may be a structural motif critical in orienting the transactivation and the DNA-binding domains of transcription factors.

This structural organization of Brachyury-like motif is also found in Arix and NBPhox, where the N-terminal activation domain and the DNA-binding homeodomain are bridged by the Brachyury-like motif. These observations suggest conservation of this structural organization across transcription factors of different classes. It would be

interesting to investigate whether there is functional modulation between Brachyury-like motif and phosphorylation of Arix, since the functional phosphorylation sites of Arix appear to locate in either the N-terminus or homeodomain. Further analyses is necessary to determine whether the Brachyury-like motif is critical for transcriptional activity of Arix.

The role of CBP and Arix in PKA-stimulated DBH transcription

Another mechanism underlying PKA-stimulated DBH transcription is recruitment of a transcriptional coactivator, CREB-binding protein (CBP). The previous studies from our laboratory demonstrated that the functional synergism of Arix with PKA involves the CBP. Substantial synergistic activation of the DBH promoter occurs in the presence of Arix, PKA, and CBP, whereas the presence of CBP elicits little or no effect on basal DBH promoter activity mediated by Arix (148). Furthermore, the N-terminal activation domain of Arix physically interacts with CBP through the third zinc-finger motif *in vitro*. Thus, CBP augments Arix's regulation of the DBH promoter in a PKA-dependent manner.

A number of transcription factors are known to interact with CBP. Some of the interactions are mediated through the third zinc finger motif of CBP, including MyoD, cFos, E1A, Ets1, and GATA1 (37, 161). The versatility of CBP in protein-protein interaction provides the mode that CBP bridges transcription factors and basal transcription machinery, resulting in the transcriptional transactivation. Similarly, the nature of interaction between Arix and CBP may function as a focal point by which multiple transcription factors distributed on the DBH promoter form multiple contacts with CBP, bridging to the basal transcription machinery and facilitating PKA-stimulated DBH transcription.

It would be interesting to examine whether the phosphorylation state of Arix influences CBP recruitment. Early studies on CBP demonstrated that activated CREB as a result of phosphorylation by PKA specifically binds to CBP (16) and enhances target gene transcription. In the case of Arix, dephosphorylated Arix upon activation of the

PKA pathway, may favorably interact with CBP. Alternatively, PKA activation may lead to phosphorylation of CBP, which contributes to PKA-stimulated augmentation of the DBH transcription. This phenomena has been observed in Pit-1, a POU-homeodomain transcription factor. Xu *et al.* demonstrated that the activation of Pit-1 by PKA was lost in the presence of CBP containing a point mutation at the consensus PKA phosphorylation site (168).

Since intrinsic histone acetyltransferase activity of CBP has been discovered, the function of CBP as a coactivator is believed to involve targeted acetylation of histone at the promoter, so that acetylated promoter region becomes more accessible to other transcription factors (7, 113). Moreover, an increasing number of transcription factors has been reported to serve as non-histone substrates of acetylation by CBP. Many of acetylatable transcription factors identified so far are specifically involved in biological functions such as DNA damage, differentiation, and development. These examples are p53 (46, 131), MyoD (132), and GATA1 (12). In all of the above examples, acetylation of transcription factors has been demonstrated *in vivo* and appears to function positively by increasing the DNA binding activity. Conversely, mutation of the lysine residues predicted to be acetylation sites impairs its transcriptional activation capacity. Furthermore, MyoD with mutations at acetylation sites fails to induce muscle differentiation. Thus, we are beginning to understand the role of acetylation of transcription factors in cellular functions. In this regard, since CBP appears to be a component of Arix-mediated DBH transcription in a PKA dependent manner, Arix is a candidate as a substrate for acetylation by CBP. It remains unexplored whether Arix's

transcriptional capacity is regulated by acetylation and whether acetylation is regulated by the phosphorylation state of Arix.

Potential Target Genes of Arix and NBPhox

Identification of the natural target genes for homeodomain proteins is often a challenging theme. In addition to TH and DBH, Arix and NBPhox are likely to regulate multiple genes involved in noradrenergic differentiation. They may also integrate with other transcription factors to direct a noradrenergic specification program. The phenotypic observation of knock-out gene analyses can hint at a couple attractive candidates; Phox2a/2b and GATA3.

Histochemical analyses in the Phox2a and Phox2b knock mice prompt us to investigate how the expression of Phox2a and Phox2b genes are regulated. It has been suggested that one of the genes initiates the expression of the other depending on types of ganglia. Moreover, no matter which gene expression starts first, the cross-regulation between Phox2a and Phox2b genes is critical to maintain each other's expression in both the CNS and PNS. Very recently, Flora *et al.* has isolated a fragment containing approximately 1.2 kb of 5'-flanking sequences from the transcription initiation site and 253 bp of transcribed sequences of human Phox2a gene (31). In their initial studies on characterization of the Phox2a promoter, one functional homeodomain recognition site was identified. This site appears to mediate the binding of Phox2b and transactivation of the Phox2a promoter by Phox2b. Further dissection of the regulatory elements in the Phox2a promoter will await to delineate the transcriptional mechanism of Phox2a in coordination with Phox2a itself, Phox2b and Mash1.

The phenotype of GATA3 deficient mice lead us to speculate its possible role in the development of noradrenergic neurons. GATA3, a zinc-finger transcription factor, is prominently expressed in hematopoietic stem cells and believed to regulate the

development of T-lymphocytes [reviewed by (77)]. It is also expressed outside the hematopoietic systems, in particular, the sympathetic ganglia and adrenal medulla (36). Targeted deletion of the GATA3 gene leads to early embryonic lethality and precludes an analyses of the developmental roles of GATA3. Further investigation revealed that the embryonic lethality of the GATA3 -/- mice is due to noradrenaline deficiency of the sympathetic nervous systems. In fact, GATA3 -/- mice can be rescued by feeding pregnant dams with dihydroxyphenylserine, a direct precursor of noradrenaline. The rescued GATA3 -/- mice interestingly displayed normal development of the sympathetic ganglia and adrenal medulla, yet, complete loss of the expression of TH and DBH in these tissues. Therefore these mice are unable to produce noradrenaline. Surprisingly, the expression of Phox2a and Phox2b are perfectly preserved in both the sympathetic ganglia and adrenal medulla. These phenotypic observation suggest that GATA3 plays a role in later development of peripheral noradrenergic neurons. The relatively broad expression pattern of GATA3 correlates far less well with the phenotype specification than that of Phox2a/2b. It is, however, possible that GATA3 participates in the maturation process of noradrenergic neurons. It still remains unsolved whether Phox2a/2b promotes the expression of GATA3, which, in turn, directly or indirectly activate the TH and DBH gene expression. Alternatively, Phox2a/2b and GATA3 may function together to maintain the expression of TH and DBH.

It should be worth noting that neuroblastoma is a disorder related to noradrenergic differentiation. Neuroblastoma, the most common extracranial solid tumor of childhood, arises from immature, undifferentiated neuroblasts of neural crest origin. Most of them occur in the adrenal medulla, while others occur in wherever the sympathetic nerves

innervate, such as abdomen, pelvis, chest, and neck. Tumors may elaborate DOPA, dopamine, and norepinephrine, causing hypertension. It is plausible that the genetic aberrations may occur in some of components involved in noradrenergic differentiation, since the biology of neuroblastoma is closely related to the developmental process of neural crest. In fact, high expression profile of FOG3, a cofactor of GATA, has been observed in a majority of neuroblastomas (114).

The last candidate target gene of Arix and NBPhox would be norepinephrine transporter (NET). NET, which is on the presynaptic membrane, uptakes norepinephrine upon synaptic transmission and terminates the action of norepinephrine. Together with DBH, NET is specifically expressed in noradrenergic neurons (92). Co-expression of DBH and NET in the nervous systems postulates that the NET gene expression may also participate in noradrenergic phenotype specification. In primary cultures of the superior cervical ganglia, leukemia inhibitory factor and ciliary neurotrophic factor, which are known to induce a switch from a noradrenergic to a cholinergic phenotype, reduce the mRNA level of NET (95). Despite the possible importance in transcriptional regulation of NET, little is known about the promoter of NET. Kim *et al.* recently has isolated 5'-upstream sequence encompassing 9.5kb from the human NET gene that drives noradrenergic cell specific transcription (67). Co-incubation of antibodies in EMSA with a probe from hNET promoter identified DNA-nuclear protein complex containing Phox2a and HoxA5 (68). However, forced expression of Phox2a failed to further activate the 9.5 kb-hNET promoter in both HeLa and neuroblastoma SK-N-BE(2)C cells (67). Similar to activation of the DBH promoter, Phox2a may require additional factors or activation of second messenger pathways to confer potentiated transcription of NET.

Further analyses are necessary to determine whether NET expression is involved in noradrenergic specification through Arix and NBPhox.

Conclusions

In the past five years, Arix and NBPhox have received increasing attention for their essential roles in noradrenergic phenotype determination. A great amounts of work have been done by employing genetic analyses in collaboration with histochemical analyses. These studies have achieved to delineate the molecular pathway of noradrenergic neuronal fate determination. However, they had a limit to understand how Arix and NBPhox exclusively activate the target genes and discriminate induction of inappropriate genes. Experiments in the present thesis applied biochemical and molecular biological techniques to overcome the above obstacles.

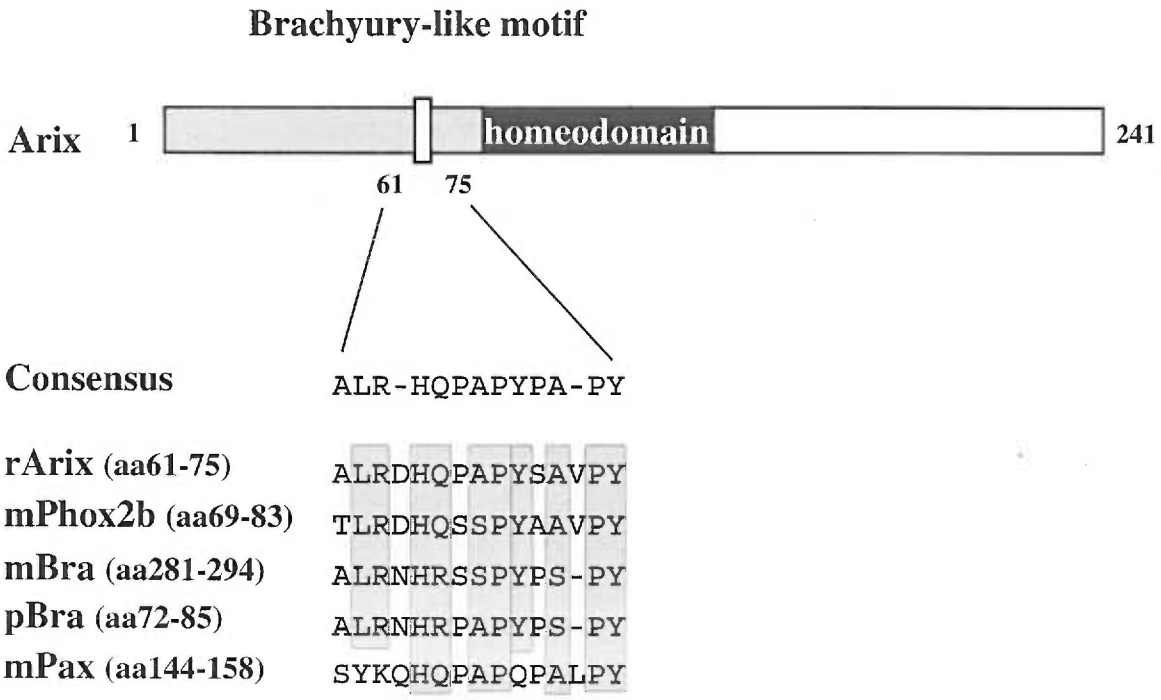
The present thesis achieved to understand the interactive mechanism between Arix and the cAMP signaling pathway toward activation of the DBH gene. The interplay between a transcription factor and a second messenger signaling is an important mechanism in a variety of biological functions. In particular, it has been hypothesized that cell fate specification is influenced by extrinsic signals during development (28). Increasing lines of evidence support the hypothesis by phenotypic observation that a particular extracellular factor induces gene expression, which, in turn, promotes a specific cell lineage. Yet, what the extracellular factor does inside a cell is largely unknown. The findings in this thesis can be applied to understand the mechanism of other transcription factors associated with cell-type specification in coordination with extrinsic signals.

Promiscuous DNA-binding specificity of homeodomain proteins *in vitro* is notorious. In the study of this thesis, we took advantage of a known natural target gene of Arix and NBPhox to understand the molecular mechanism underlying activation of the DBH gene transcription. The present studies do not rule out whether dephosphorylation

of Arix is necessary to discriminate the activation of non-specific genes. Nevertheless, the findings have potential to speculate that the mode of post-translational modification of homeodomain proteins is one way to specify the target gene activation. Understandings of the molecular mechanism of Arix and NBPhox will also shed lights on how other homeodomain transcription factors activate particular target genes in a discriminatory fashion.

Figure 4.1. An alignment of homologous segments of a Brachyury-like motif contained within the N-terminal activation domain of Arix (residues 61-75). This motif was found in the Arix family member, NBPhox/Phox2b, the T-box transcription factor, Brachyury, and the paired-box transcription factor, Pax9. The Brachyury-like motif is conserved in transcription factors from several divergent classes and may have relevance in activation domain function. Presented for alignment are the sequences for rat Arix, mouse Phox2b, pig and mouse Brachyury, and mouse Pax9.

Figure 4.1


Brachyury-like motif
Arix
1
homeodomain
241
61
75
Consensus
ALR-HQPAPYPA-PY
rArix (aa61-75)
ALRDHQPAPYSAVPY
mPhox2b (aa69-83)
TLRDHQSSPYAAVPY
mBra (aa281-294)
ALRNHRSSPYPS-PY
pBra (aa72-85)
ALRNHRPAPYPS-PY
mPax (aa144-158)
SYKQHQPAPQPALPY

Appendix I

Toward Identification of

Functional Phosphorylation Sites of Arix

Minimal Domains Required for Protein-Protein Interaction of Arix

Arix and NBPhox have the ability undergo homo- and heterodimerization *in vitro* (1) and *in vivo* (148). In order to understand the functionally important domains of Arix, we have initiated to map out a minimal domain necessary for the protein-protein interaction. We also asked whether the minimal domain preferably interacts in either the phosphorylated or the dephosphorylated state. As described in Chapter 3, ^{35}S-labeled IVT proteins were subjected to the kinase reaction with PC12 nuclear extracts to produce phosphorylated proteins (Fig. A.1a). Consistent to the results (Fig. 3.6) in the Chapter 3, phosphorylated proteins were obtained from ArΔN and HDAr constructs in this kinase assay system. The phosphorylated products were then used for His pull-down assays to evaluate the protein-protein interaction. His-Arix was able to interact strongly with both, ArΔC and HDAr, and weakly with ArΔN construct, suggesting that the homeodomain of Arix is sufficient to interact with other proteins and that the C-terminal segment of Arix may have an inhibitory role in the protein interaction. Furthermore, these protein-protein interactions through the homeodomain Arix occurred preferably in the dephosphorylation state since the faster migrating proteins, which are presumably phosphorylated proteins, were primary His-Arix interacting proteins (Fig A.1b). In contrast, the slower migrating proteins were abundantly present in an aliquot of supernatant after the binding reaction. The protein-protein interactions with ArΔC and HDAr were still evident even after the micrococcal nuclease treatment, excluding the possibility that the interaction is non-specifically mediated by association of DNA (Fig A.1c). Thus, homodimerization of Arix appears to be mediated through the homeodomain and is preferred in the dephosphorylation state. Since NBPhox has an identical homeodomain,

homodimerization of NBPhox as well as heterodimerization with Arix are likely to be mediated through the homeodomain.

The Analyses of Predicted Phosphorylation Sites in The Arix Homeodomain

To further elucidate the mechanism involved in the PKA-regulated activity of Arix, we began to seek the functionally responsible phosphorylation sites on Arix. Consensus amino acid sequence analyses revealed that multiple phosphorylation sites are present in the C-terminus as well as in the homeodomain, but none in the N-terminus (Fig. I-2A). These are targets for tyrosine kinase, casein kinase, PKC, and MAPkinase. No PKA phosphorylation sites were predicted. The previous results in Chapter 3 showed that activation of PKA appears to affect the DNA binding activity of Arix (Fig. 3.4) and that the homeodomain of Arix mediates the protein-protein interaction (Fig. A.1). We, therefore, first focused on the possible phosphorylation sites within the homeodomain, the DNA binding motif: tyrosine25 (Y25) and threonine30 (T30) residues by tyrosine and casein kinases, respectively.

In order to predict the spatial location of the side chains of these residues, we designed the structure of Arix homeodomain, using a known structure of homeodomain. We chose Paired as a comparison, which shares 70% amino acid identity with the homeodomain of Arix; therefore, it is reasonable enough to predict the structure of the Arix homeodomain. The designed structure of the Arix homeodomain demonstrated a classical helix-turn-helix motif composed of helices 2 and 3. The Y25 and T30 residues are located on the loop between helix1 and 2 and on the helix3, respectively (Fig. A.2b). Importantly, the side chain of the Y25 extends to the backbone of DNA (Fig. A.2c). The closest distance between OH group of the Y25 and the phosphate group of DNA was predicted 2.4Å, a reasonable distance to form a hydrogen bond. It is likely that

modification by the negatively charged phosphorylation at Y25 becomes repulsive and too bulky to make a contact with the backbone of DNA, disrupting binding of Arix to DNA. Thus, it is possible that dephosphorylation of the Y25 may be functionally critical to activate Arix. Another potential phosphorylation site, T30, is on the helix2. The OH group of the T30 does not appears to make a direct contact with DNA (Fig. A.2d). Instead, the OH group is likely to have accessibility to interact with a kinase or a phosphatase for post-translational modification.

Based on the results from Chapter 3 as well as the above structural prediction, we hypothesized that dephosphorylated Arix is transcriptionally active and that either the Y25 or T30 residue would be the functional target phosphorylation sites. In order to test the hypothesis, the Y25 and T30 residues were mutated to non-phosphorylatable residues, phenylalanine and alanine, respectively. The transcriptional activities of these mutant Arix constructs were examined using the DBH-reporter promoter construct in HepG2 cells. We anticipated that the mutant Arix constructs would be able to strongly activate the DBH promoter even without the stimulation of the PKA pathway. However, the results were puzzling: activities of the Y25F mutant was as equal as the wild-type (wt) Arix in the absence of PKA stimulation, while the T30A mutant resulted in a slight increase in the DBH promoter activity without PKA stimulation. In the Y25F mutant, PKA-stimulated DBH transcription demonstrated a 25% decrease compared to the wt Arix. When the fold increase in PKA-responsiveness on the DBH transcription was compared between wild-type and mutant constructs, both Y25F and T30A mutant Arix substantially reduced the PKA-responsiveness by 55-65% in comparison to the wt Arix.

We also tested the DNA-binding activities of the above mutant Arix constucts by EMSAs, using 293 cell extracts overexpressing one of the Arix constructs. The protein content of mutant and wt Arix proteins were roughly equal in the cell extracts under both basal and forskolin-stimulated conditions as shown by western blots with the HA antibody (Fig. A.4a). Similar to the EMSA (Fig. 3.4) in the previous chapter, the extent of supershift formation with the HA antibody is used to evaluate the DNA binding activity in Fig. A.4b. Surprisingly, the DNA-binding activities of the Y25F and T30A mutants were almost absent with the DB1 and HD3 probes, even cells were under the PKA-stimulated condition, suggesting that the Y25 and T30 residues are involved in DNA binding either directly or indirectly. Replacement of the Y25 residue with phenylalanine leads to the absence of a hydroxyl group, which is a critical side chain to contact with the backbone of DNA through a hydrogen bond as predicted from the structural analyses (Fig. A.2c). Thus, the inability of the Y25F mutant to bind to DNA may result from the loss of the hydroxyl group. The mechanism by which the T30A mutant Arix lose the DNA-binding activity is unclear. Since the T30 residue is on the surface of the helix2, the side chain of T30 residue may be involved in the interaction with other protein that stabilize the DNA binding of Arix. Mutation with alanine residue may interfere the protein interaction.

Summary

In some classes of homeodomain proteins, protein-protein interactions with other homeodomain protein or other types of transcription factors confer functional significance, including transcriptional synergism through cooperative binding and sequence specificity for target DNA binding. In relation to our work, cooperative interaction involving homeodomain proteins have been documented in Phox1 and Pbx1. Phox1 shares high amino acid homology with Arix and NBPhox within the homeodomain. Phox1 interacts with non-homeodomain proteins, serum response factor (SRF), enhancing the DNA-binding activity of SRF to the serum responsive element (SRE), therefore, increasing transcription of cFos, the down-stream gene of SRE (45). In addition, the homeodomain of Phox1 is sufficient for the interaction with SRF to achieve the cooperative DNA-binding activity. In the case of homeodomain protein Pbx1, it has been demonstrated to interact cooperatively with other homeodomain proteins, Hox proteins (14, 93, 111). The homeodomain of Pbx1 is sufficient for the cooperative DNA-binding with a Hox protein. Furthermore, detailed point mutational analyses of the Pbx1 homeodomain suggested that the loop between helices 1 and 2 and amino acid resides on helix2 of Pbx1 contributes to the cooperative interaction with Hox A5. This loop is predicted to interact with the N-terminal arm of the homeodomain of Hox A5. Since the recognition helix of Hox2A (helix 3) lies beneath the surface of the helix 2 of Pbx1, the amino acid residues on helix2 of Pbx1 could support the protein interaction with Hox2A.

I have demonstrated that Arix and NBPhox can also form homo- or heterodimers. As demonstrated in Phox1 and Pbx1, it is not surprising that the homeodomain of Arix mediates not only DNA-binding but also protein-protein interaction. In particular,

dimerization of Arix and NBPhox preferably occurs under dephosphorylated conditions. It is intriguing to investigate whether this protein-protein interaction is functionally important to regulate DBH gene transcription *in vivo* in collaboration with phosphorylation states of Arix and NBPhox.

Collectively, the mutations at the Y25 and T30 residues in the homeodomain of Arix impair the DNA-binding activity, yet these mutant constructs are still able to activate the DBH promoter. These results imply that Arix may have an ability to recruit other transcription factors or coactivators without tightly binding to DNA, resulting in acitvation of the DBH promoter. Failure to detect tyrosine phosphorylation of Arix by the 2-D phospho-amino acid mapping is notorious (Fig. 3-6). It is still inconclusive whether the Y25 residue is phosphorylated *in vivo* and whether it is the functionally responsible residue in response to activation of the PKA pathway. As suggested by phospho-amino acid mapping, we can not exclude the possibility that serine residues present in the N-terminus or the homeodomain are target phosphorylation sites. More extensive analyses at each amino acid level are necessary to identify the functional phosphorylation sites.

Figure A.1 A minimal domain for protein-protein interaction of Arix is the homeodomain and occurs preferably in a dephosphorylated form. (A) Truncated Arix constructs were *in vitro* translated (IVT) and ^{35}S-labeled and subject to *in vitro* kinase assay using PC12 nuclear extracts as a source of kinases. The reaction products were resolved on SDS-PAGE followed by autoradiograph. The truncated Arix constructs used in the experiments are depicted in Fig 3-6 in the Chapter 3. **(B)** In combination with kinase assays, truncated Arix constructs were analyzed for *in vitro* protein-protein assays. Prior to protein-protein interaction assays, ^{35}S-IVT proteins were treated with PC12 nuclear extracts in the kinase buffer. All reaction products in the kinase assay were then incubated with TALON metal resin in the presence of recombinant His-Arix. After the binding reactions, resin was washed and His-Arix interacting proteins were analyzed by SDS-PAGE, followed by autoradiography. Sup represents unbound fractions collected from the supernatant of the binding reaction before washing TALON metal affinity resin. In all cases, phosphorylated ^{35}S-IVT products were abundant in the unbound fraction. Some reactions included PAPase during kinase assays to dephosphorylate the phosphorylated products. **(C)** After the same *in vitro* binding assays described above, washed resin was further treated with micrococcal nuclease prior to SDS-PAGE analyses. The binding assays with and without micrococcal nuclease treatment were carried out in parallel and examined by autoradiography with an exactly same exposure time.

Figure A.1

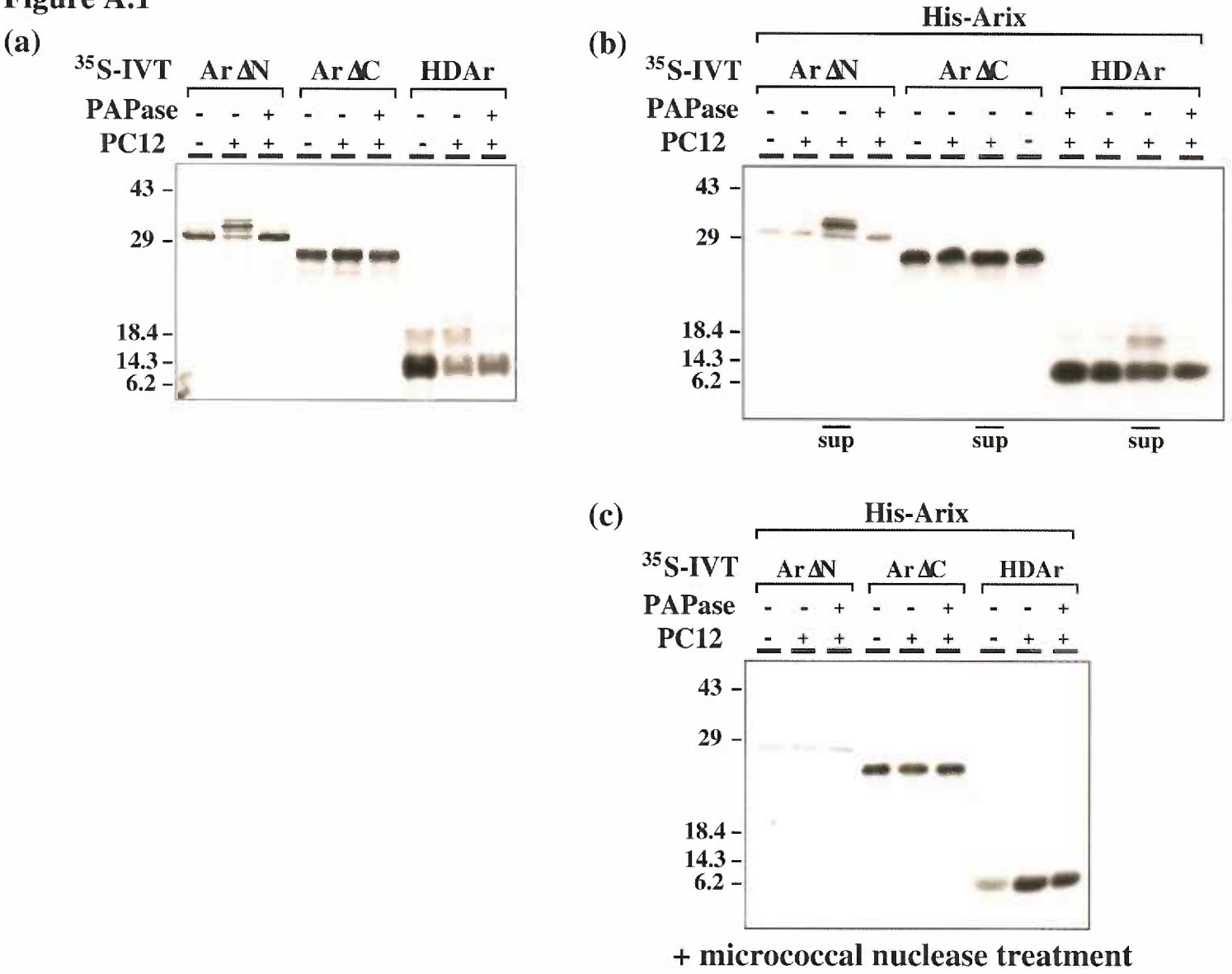
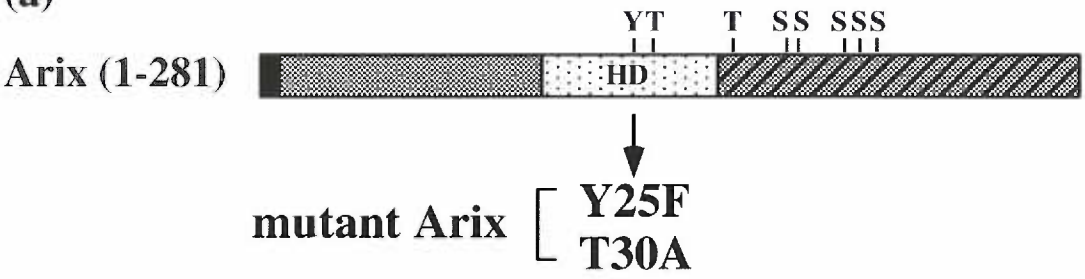

(a)
35S-IVT
Ar ΔN
Ar ΔC
HDAr
PAPase
PC12
43
29
18.4
14.3
6.2
(b)
His-Arix
35S-IVT
Ar ΔN
Ar ΔC
HDAr
PAPase
PC12
43
29
18.4
14.3
6.2
sup
sup
sup
(c)
His-Arix
35S-IVT
Ar ΔN
Ar ΔC
HDAr
PAPase
PC12
43
29
18.4
14.3
6.2
+ micrococcal nuclease treatment

Figure A.2 The tyrosine residue (Y25) in the homeodomain is a key residue to make a contact with DNA. **(A)** Based on the consensus amino acid sequences, possible phosphorylation sites are depicted on the diagram of Arix. Multiple phosphorylation sites are present on the C-terminus to the homeodomain. In addition, the homeodomain contains two possible phosphorylation sites, tyrosine 25 (Y25) and threonine 30 (T30) resides, which are replaced with phenylalanine and alanine, respectively, for the following experiments. **(B)** The structure of Arix homeodomain was designed based on the crystal structure of Paired, which shares 70% identity with Arix in the homeodomain. Using the XtaI View program (98), coordinates necessary to design the structure of Arix were obtained. Helices 2 and 3 form the helix-turn-helix DNA binding motif, in which helix3 lies in the major groove of DNA. The side chains of Y25 and T30 are presented in green and orange, respectively. **(C)** The hydroxyl group of the Y25 reside contacts with the phosphate backbone of DNA by forming a hydrogen bond. **(D)** The hydroxyl group (indicated by red stick and ball) of the T30 reside sticks out to the surface; thereby, available to access with a kinase and/or a phosphatase.

Figure A.2

(a)



(b)

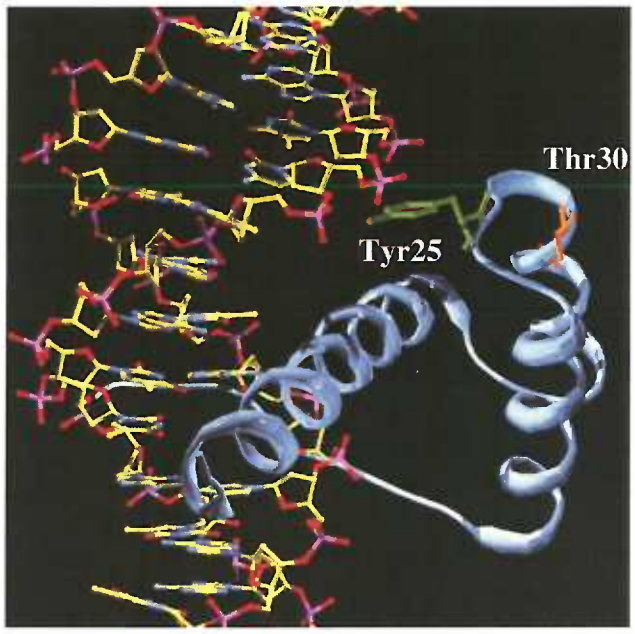
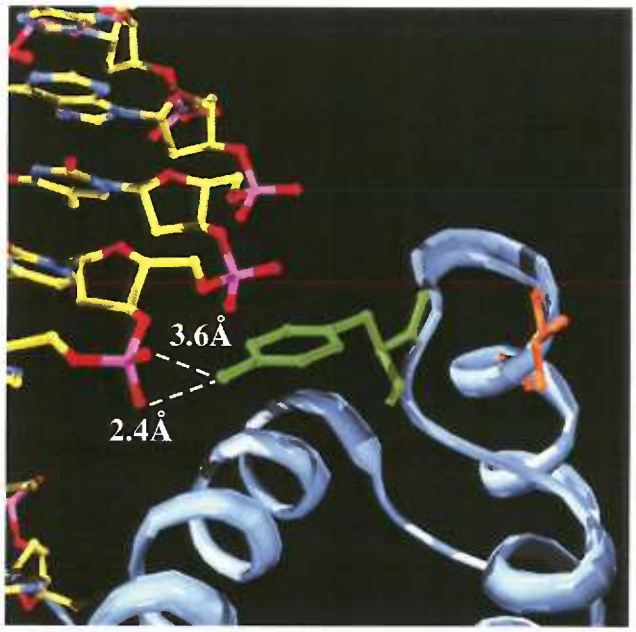


Figure A.2

(c)



(d)

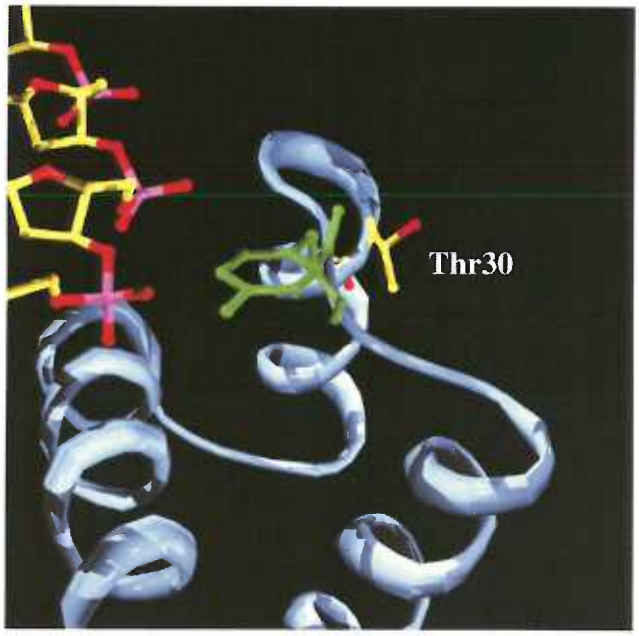


Figure A3 Y25 and T30 Mutations in the homeodomain leads to the reduction in the PKA-responsiveness on the DBH transcription. HepG2 cells were transfected with DBH-Luc reporter, pRL-null, either wild type (WT) or mutant Arix (Y25, T30) construct and either with or without PKA expression vector. HA6.1 is a backbone of the Arix expression plasmid. Values for firefly luciferase activity were normalized to those for the renilla luciferase activity and calculated relative to the basal promoter activity in the absence of Arix and PKA. Thus, the mean values of fold luciferase activity for the basal control is equal to 1. Bars present mean + SD from one representative experiment. Experiments were repeated three times with similar results. To evaluate the PKA-responsiveness, the ratio of normalized DBH-Luc activity under PKA-stimulated condition to that under the basal condition (-PKA) for each Arix construct was calculated.

Figure A.3

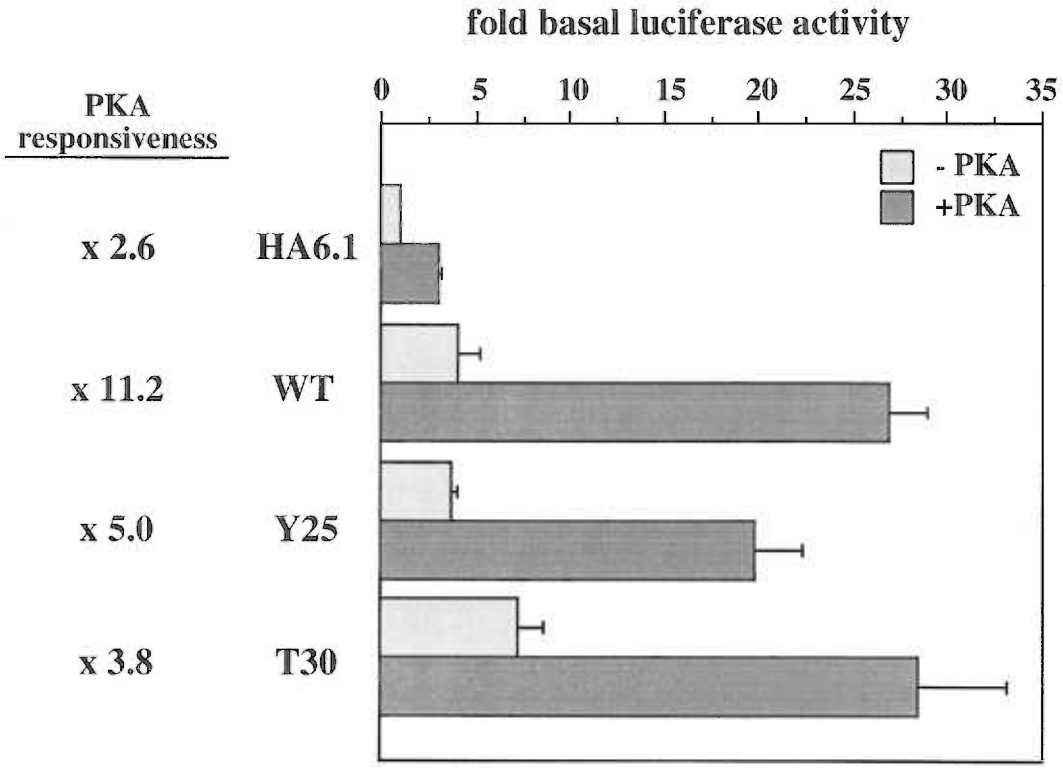
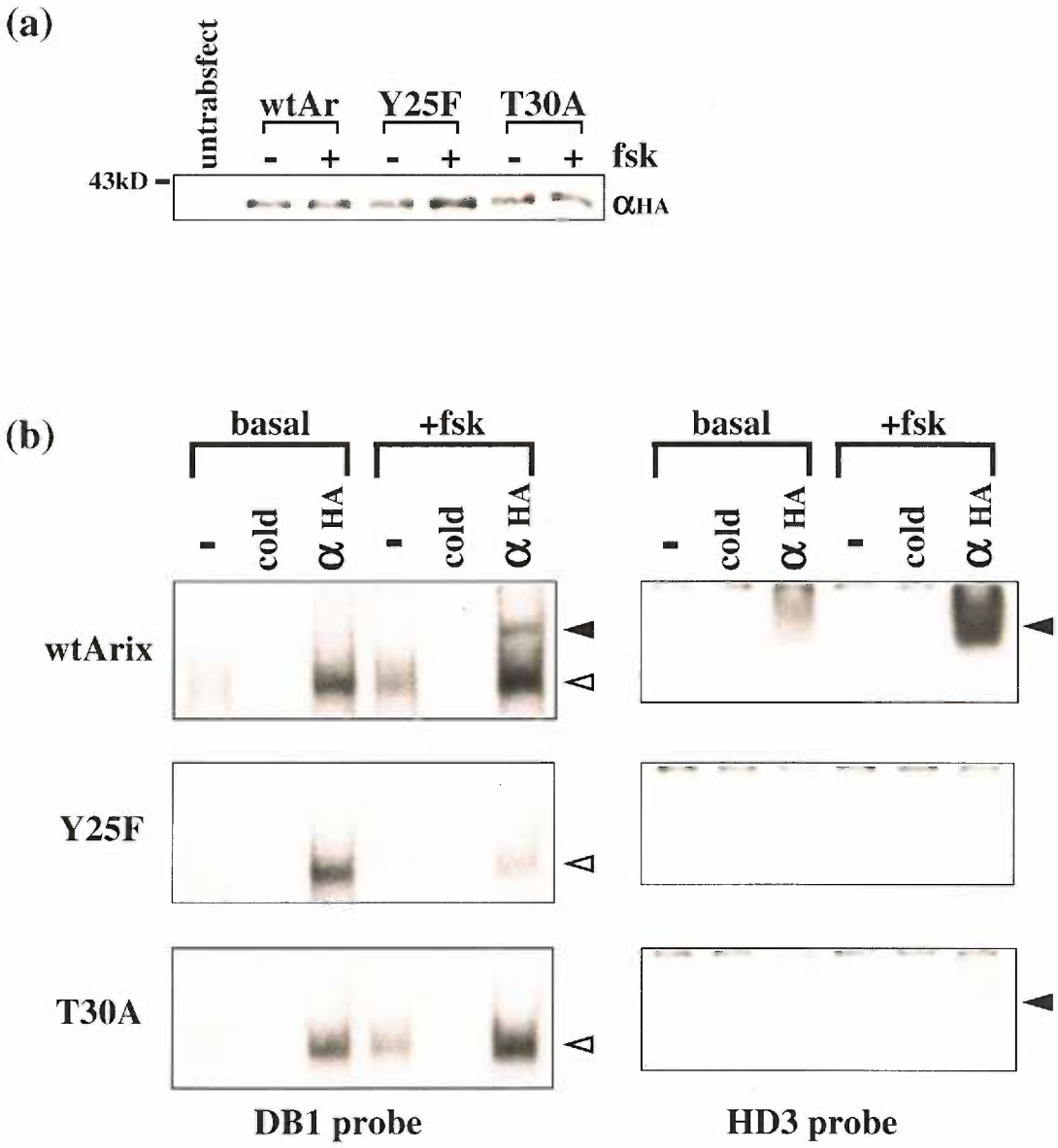

fold basal luciferase activity
0
5
10
15
20
25
30
35
PKA responsiveness
- PKA
+PKA
x 2.6
HA6.1
x 11.2
WT
x 5.0
Y25
x 3.8
T30

Figure A.4 Y25F and T30A mutant Arix lose their DNA-binding activity. (A) HEK293 cell were transiently transfected with 5μg of either HA-Arix or mutant Arix construct. One day after transfection, cells were stimulated with fsk for 1hr and harvested for the preparation of cell extracts. The contents of wild-type and mutant Arix in HEK293 cell extracts were then examined by a western blot. 2.5μg of cell extracts were separated on a 10% SDS-PAGE and HA-tagged Arix was stained with the HA antibody. **(B)** Using HEK293 cell extracts described in (A), EMSA was carried out with DB1 and HD3 probes. As a competition assay of EMSA, 50ng of an unlabeled oligonucleotide was added in the binding reaction (cold). To evaluate the DNA binding activity of Arix, 0.5μg of HA antibody was co-incubated (αHA). The formation of a supershift complex was observed from the wild-type Arix with both DB1 and HD3 probes, but not from the mutant Arix, as indicated by closed arrowheads. The mobility shifts indicated by open arrowheads are also observed in untransfected extracts (data not shown) and are non-specific DNA-protein complexes.

Figure A.4


(a)
untrabsfect
wtAr
Y25F
T30A
- + - + - +
fsk
43kD
αHA
(b)
basal
+fsk
basal
+fsk
- cold α HA - cold α HA
- cold α HA - cold α HA
wtArix
Y25F
T30A
DB1 probe
HD3 probe

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