Protein-Protein Interactions and Cell Signaling in the Regulation of HOX•PBX Functions

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ABSTRACT

HOX proteins are homeodomain-containing transcription factors essential for embryonic patterning. Despite amino acid differences, all HOX homeodomains recognize highly similar sites on DNA. One mechanism by which HOX proteins achieve specificity is through interaction with cofactors of the PBX and MEIS/PREP1 families. Higher order complexes between HOX, PBX and MEIS/PREP1 proteins form *in vivo* and are essential for target recognition and transcriptional regulation. Another level of control of HOX function is the nuclear availability of its cofactors. This thesis addresses the regulation of the nuclear availability of the PBX protein by MEIS/PREP1 family members. We identified two nuclear localization signals (NLS) in the PBX homeodomain and showed that the NLS are masked in the absence of MEIS/PREP1. Upon a conformational change in PBX induced by MEIS/PREP1 binding, the NLS are exposed and a receptor-mediated active transport of PBX into the nucleus is allowed. This thesis also investigates the mechanisms of transcriptional regulation by the HOX•PBX complexes. We show that HOX•PBX complexes repress transcription and are switched to transcriptional activators in response to cell signaling. We demonstrate that PBX mediates the repression function by recruiting histone deacetylases (HDACs) to HOX target promoters. Inhibition of HDAC activity or stimulation of protein kinase A (PKA) signaling converts the HOX•PBX complex into a net activator of transcription. The activation function is mediated by the HOX protein through its recruitment of CREB-binding protein (CBP), a coactivator with histone acetyl-transferase (HAT) activity. We propose a model whereby HOX•PBX transcriptional activity is determined by cell signaling, and is mediated by the local modification of chromatin structure in the promoter of downstream targets.

RESUMÉ

Les protéines HOX sont des facteurs de transcription contenant un domaine homéotique et sont essentiels pour le "patterning" de l'embryon. Malgré des différences en acides aminés, les domaines homéotiques des protéines HOX reconnaissent des sites similaires de liaison à l'ADN. Un mécanisme par lequel les protéines HOX accomplissent leur spécificité est l'interaction avec des cofacteurs des familles PBX et MEIS/PREP1. Des complexes tripartites entre les protéines HOX, PBX et MEIS/PREP1 ont été observés *in vivo* et démontrés essentiels pour la reconnaissance de gènes cibles et leur régulation transcriptionnelle. Un autre niveau de contrôle des fonctions de HOX est la présence de ses cofacteurs dans le noyau. Cette thèse porte sur la régulation de la localisation nucléaire des protéines PBX par les membres de la famille MEIS/PREP1. Nous avons identifié deux signaux de localisation nucléaire (NLS) dans le domaine homéotique de PBX et démontré que les NLS de PBX sont masqués en absence de MEIS/PREP1. En conséquence d'un changement de conformation dans PBX induit par l'interaction avec MEIS/PREP1, les NLS sont exposés et un transport actif de PBX dans le noyau est permis. Cette thèse explore aussi les mécanismes de la régulation de la transcription par les complexes HOX•PBX. Nous montrons que les complexes HOX•PBX répriment la transcription et sont convertis en activateurs de la transcription en réponse aux signaux cellulaires. Nous démontrons que PBX cause la répression en recrutant des déacétylases de histones (HDACs) sur les promoteurs cibles de HOX. L'inhibition de l'activité HDAC ou la stimulation de la protéine kinase A (PKA) convertissent le complexe HOX•PBX en un activateur de la transcription. La fonction d'activation est portée par la protéine HOX qui recrute la « CREB-binding protéine »

(CBP), un coactivateur muni d'une activité HAT. Nous proposons un modèle dans lequel l'activité transcriptionnelle de HOX•PBX est déterminée par les signaux cellulaires et est causée par des modifications locales de la structure de la chromatine au niveau des promoteurs des gènes cibles.

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Mom, Dad, Wassim and Walid, I hope that one day you would read my thesis and see how much I appreciate your presence in my life. I thank you for everything I have accomplished and for all what I have today.

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PREFACE

This thesis is presented in manuscript-based form, in accordance with section 2 of the "Guidelines for Thesis Preparation" established by the Faculty of Graduate Studies and Research of McGill University, as cited below:

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the Thesis Preparation Guidelines with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

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In the case of manuscripts co-authored by the candidate and others, "the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these

cases, it is in the candidates's interest to make perfectly clear the responsibilities of all authors of the co-authored papers".

Contributions of Authors

CHAPTER II: Saleh, M., Huang, H., Green, N. C., and Featherstone, M. S. (2000a). A conformational change in PBX1A is necessary for its nuclear localization, Experimental Cell Research *260*, 105-115.

All the figures in this paper were done by myself, except for figure 7 that was provided by He Huang. Nancy Green had the initial observation of a better binding by Δ1-232, presented in part of figure 4A. Dr. Mark Featherstone supervised the work presented in this paper.

CHAPTER III: Saleh, M., Rambaldi, I., Yang, X. J., and Featherstone, M. S. (2000b). Cell signaling switches HOX-PBX complexes from repressors to activators of transcription mediated by histone deacetylases and histone acetyltransferases, Molecular and Cellular Biology *20*, 8623-8633.

All the figures in this paper were done by myself. Isabel Rambaldi had the initial observation of the effect of PKA on HOXD4 transactivation, presented in part of figure 5B. Dr. Xiang-Xiao Yang co-supervised the work presented in this paper along with Dr. Mark Featherstone.

Original Contributions to Knowledge

1. Demonstrated that all MEIS/PREP1 family members direct PBX1A to the nucleus.
2. Demonstrated that an N-terminal region of PBX1A (residues 172-219) is inhibitory for its nuclear localization.
3. Identified two nuclear localization signals (NLS) in PBX1A and mapped them to the N-terminal arm and the third α-helix of its homeodomain.
4. Demonstrated that the two NLS are required to direct PBX1A to the nucleus and that their function is cooperative.
5. Demonstrated that PBX1A NLS are sufficient for nuclear localization induced by PREP1.
6. Demonstrated the existence of intramolecular interactions in PBX1A that block its NLS function.
7. Identified a mutation in the PBX1A homeodomain (E28R) that changes the conformation of the protein and results in its constitutive nuclear localization.
8. Demonstrated that HOX•PBX complexes repress transcription in the absence of activating signaling cues.
9. Demonstrated that PBX1 associates with class I histone deacetylases (HDAC1 and HDAC3) *in vivo via* its second N-terminal repression domain (residues 89-172).
10. Demonstrated that the first repression domain in PBX1A (residues 1-89) functions in a TSA-insensitive manner.

11 Demonstrated that PBX1 recruits the corepressor N-coR and SMRT to repress transcription of HOX•PBX-responsive enhancers.

12 Demonstrated that mSIN3B but not mSIN3A is part of the N-coR/HDAC corepressor complex *in vivo*.

13 Demonstrated that HOXD4 associates with the coactivator CBP *via* its N-terminal activation domain to activate transcription.

14 Demonstrated that the histone acetyltransferase (HAT) domain in CBP is involved in its interaction with HOXD4.

15 Demonstrated that protein kinase A (PKA) signaling superactivates HOX•PBX-responsive enhancers and that CBP transduces the PKA effect.

16 Demonstrated that HOX•PBX complexes are switched from repressors to activators of transcription in response to inhibition of HDACs, stimulation of PKA signaling, or by signaling pathways induced by cellular aggregation.

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LIST OF ABBREVIATIONS

aa: amino acids
ABD-B: abdominal-B
ACF: ATP-utilizing chromatin assembly and remodeling factor
ACTR: activator of thyroid and retinoid receptors
AER: apical ectodermal ridge
ALL; acute lymphoblastic leukemia
ANT-C: antennapedia complex
A/P: anterior/posterior
ARC: activator-recruited cofactor
ARE: autoregulatory element
BCD: bicoid
bHLH: basic helix-loop-helix
bp: base pair
BRM: brahma
BX-C: bithorax complex
C1: cervical vertebra 1
CAM: cell adhesion molecules
CAS: cellular apoptosis suceptibility
CBP: CREB-binding protein
C/H3: cystidine/histidine-rich domain
CHRAC: chromatin remodeling activity
CMV: cytomegalovirus
CNS: central nervous system
CRABP: cellular retinoic acid binding protein
CREB: cyclic AMP-response element-binding protein
CRM-1: chromosome maintenance 1
CRSP1: cofactor required for SP1 activation
CTD: Carboxy-terminal domain
DAC: dachshund
DBD: DNA-binding domain
dCBP: *Drosophila* CBP
Dfd: deformed
Dll: distalless
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
dpc: days post coitum
DPP: decapentaplegic
DR2/5: direct repeat 2/5
DRIP: vitamin D3 receptor interacting protein
E1A: early region 1A
E2A:early region 2A
EC: embryonal carcinoma
EGL-27: egg-laying
Egr2: early growth response 2

EKLF: erythroid kruppel-like factor
EM: electron microscopy
EMSA: electromobility shift assay
EN: engrailed
ER: estrogen receptor
EXD: extradenticle
FCS: fetal calf serum
FGF: fibroblast growth factor
FKH: forkhead
FTZ: fushi tarazu
GDP/GTP: guanosine di/tri-phosphate
GFP: green fluorescent protein
GTFs: general transcrption factors
HA: hemagglutinin
HAT: histone acetyl-transferase
HB: hunchback
HD: homeodomain
HDA1: histone deacetylase 1
HDAC: histone deacetylase
HEK293: human embryonic kidney 293
HH: hedgehog
HMG1: high mobility group 1
HTH: Homothorax
IBB: importin β-binding domain
IP: immunoprecipitation
kb: kilobase
K_D: equilibrium dissociation constant
KDa: kilodalton
LAB: labial
LIN39: lineage protein 39
LMB: leptomycin B
LTR: long terminal repeat
Mat: mating type
MDa: megadalton
MED: mediator
MEIS: myeloid ecotropic viral insertion site
MOZ: monocytic leukemia zinc finger protein
mRNA: messenger RNA
MTA-1: metastasis-associated factor 1
MYST: MOZ-YBF2/SAS3-SAS2-Tip60
NAD: nicotinamide adenine dinucleotide
NCC: neural crest cells
N-CoR: nuclear receptor corepressor
NE: nuclear envelope
NES: nuclear export signal
NLS: nuclear localization signal

NMR: nuclear magnetic resonnance
NPC: nuclear pore complex
N-terminal: amino-terminal
NTF-2: nuclear transport factor 2
NURD: nucleosome remodeling and deacetylation complex
NURF: nucleosome remodeling factor
Nup: nucleoporins
PBS: phosphate buffered saline
PBX: pre B cell ALL-related factor
P/CAF: p300/CBP-asociated factor
PCE: PBC cooperativity element
PcG: polycomb group
PCR: polymerase chain reaction
PDX-1: pancreatic and duodenum factor 1
PHO: pleiohomeotic
PIC: pre-initiation complex
PIT-1: pituitary protein 1
PKA: protein kinase A
PKI: inhibitor of PKA
POLII: RNA polymerase II
PP2A: protein phosphatase 2A
pRB: retinoblastoma protein
PRE: polycomb response element
PREP1: PBX-regulatory protein 1
PRS: PBX response sequense
r: rhombomere
RA: retinoic acid
RALDH2: retinaldehyde dehydrogenase
RAN: RAS-related nuclear protein
RAN BP: RAN binding protein
RAN GAP: RAN GTPase activating protein
RAN GEF: RAN guanine exchange factor
RAR: retinoic acid receptor
RARE: retinoic acid receptor response element
RID: receptor interacting domain
RNA: ribonucleic acid
RSC: remodel the structure of chromatin
RSV: rous sarcoma virus
RTS: Rubintein-Taybi syndrome
RXR: retinoic acid related receptor
S2: Schneider's cells
SCR: sex comb reduced
SDS-PAGE: sodium dodacyl sulfate polyacrylamide gel electrophoresis
SHH: sonic hedgehog
SIN3: switch independent
SIR: silent information regulator

SMRT: silencing mediator (corepressor) for retinoid and thyroid hormone-receptors
SNF: sucrose non fermenting
SRC: steroid receptor coactivator
SREBP1: sterol response enhancer binding protein 1
SV40: Simian virus 40
SWI: switch
T2: thoracic vertebra 2
TAF: TFIID-associated factor
TALE: three aa loop extension
TBP: TATA binding protein
TCF: T cell factor
Tip60: HIV-1 Tat interactive protein 60
TOT: α-hydroxytamoxifen
TRAP: thyroid receptor associated protein
tRNA: transfer RNA
TrxG: trithorax group
TSA: trichostatin A
UBX: ultrabithorax
VP16: viral protein 16
WCE: whole cell extract
WG: wingless
YY1: Yin Yang 1

CHAPTER I

INTRODUCTION

1. GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

HOX proteins are homeodomain-containing transcription factors essential in specifying relative positional identity along the anterior/posterior (A/P) body axes during embryonic development. In addition, HOX proteins as well as their DNA-binding partners (members of the PBX and MEIS/PREP1 families) are implicated in various human diseases including leukemias of the lymphoid lineages. It is therefore essential to understand how HOX•cofactor complexes specifically function to differentially regulate the transcription of downstream targets and control events such as cellular differentiation and, more globally, embryonic patterning.

In the first chapter, I will present a brief introduction about the discovery of the *Hox* genes, their evolution, their patterns of expression and the regulatory mechanisms controlling their precise spatio-temporal expression. In addition, I will review in more detail the current state of knowledge regarding the HOX protein as a transcription factor, its DNA-binding partners and their roles in conferring specificity to the HOX protein. Additional functions of the DNA-binding partners, such as the control of PBX nuclear availability by the MEIS/PREP1 proteins, will be addressed as a prelude to evidence documenting the mechanisms of such a regulation in chapter 2. The signaling pathways that regulate HOX functions and the downstream targets of the HOX proteins will be reviewed as well to introduce the objective of the study presented in chapter 3.

Chapter 3 proposes a model implying cell signaling as a direct determinant of HOX•PBX function in the patterning of the animal embryo. In response to signaling cues, HOX•PBX complexes are switched from repressors to activators of transcription through the differential recruitment of corepressor and coactivator complexes, respectively. Such

coregulators act by locally modifying chromatin structure *via* histone deacetylase or acetyl transferase activities.

Conclusions drawn from the experimental results will be summarized in chapter 4. Several points of interest arising from the study of PBX subcellular regulation by MEIS/PREP1 will be addressed and relevant future experiments will be proposed. Future directions toward a better understanding of the transcriptional regulation by HOX in response to different signaling pathways will be suggested and experiments that further investigate the role of chromatin structure in this regulation will be proposed.

2. THE MAMMALIAN HOX FAMILY: AN OVERVIEW

What tells a leg to be a leg or an ear to be an ear? What transform multiplying embryonic cells into spatially ordered differentiated cells? What are the signals responsible for providing a cell with its positional information? What are the consequences of a misinterpretation of such signals? And what are the downstream effectors of the patterning signals? Such fundamental questions have intrigued developmental biologists for years, and continue to do so, and are what led to the discovery of the *Hox* family.

The term **homeosis** was defined over 100 years ago by William Bateson as a morphological change where "something has taken the identity of something else" (Bateson, 1894). The first **homeotic transformation** was observed in a *Drosophila* mutant, *bithorax*, in which the third thoracic segment (T3) of the fly body was transformed into the identity of the second thoracic segment (T2). As a result, the adult fly had an extra set of wings, a T2 structure, instead of the halteres (small balancers), the

normal structures that derive from T3, resulting in a four-winged fly (Lewis, 1994 and references therein). Several of such homeotic mutations were generated and the genes harboring the mutations were termed **the homeotic genes**. E.B. Lewis, analyzing various homeotic mutants, organized the *Drosophila* homeotic genes in tandem and defined the *bithorax* complex (BX-C) (Lewis, 1978).

It was not until the establishment of various molecular tools that the homeotic genes in *Drosophila* were cloned by chromosome walking (Garber et al., 1983; Scott et al., 1983). Cross-hybridization between different *Drosophila* homeotic genes revealed a conserved 180 bp sequence that was termed the **homeobox** (McGinnis and Krumlauf, 1992). The homeobox encodes a 60 amino acid DNA-binding domain called the **homeodomain**. The demonstration of sequence-specific DNA-binding by the homeodomain suggested that homeoproteins are transcription factors (for review, see Scott et al., 1989). Using low stringency cross-hybridization screens with the *Drosophila* homeotic genes homeodomain as a probe, Homeobox-containing genes were rapidly cloned in other species including *Xenopus*, mice and man (Carrasco et al., 1984; McGinnis et al., 1984). Today, the nomenclature ***Hox*** refers to those homeobox-containing genes that are evolutionarily grouped in conserved clusters and are related to the *Drosophila* homeotic genes. *Hox* genes are found in all animals studied ranging from *Hydra*, *C. elegans*, crustaceans, primitive chordates to all vertebrates (Krumlauf, 1994). This led S. Carroll to state that "all animals, share a conserved family of genes, the *Hox* genes, important for **body patterning**" (Carroll, 1995) and Slack *et al.* to define an animal by "an organism that bears a *Hox* gene" (Slack et al., 1993).

2.1 Genomic organization and evolution of the mammalian *Hox* genes

In mammals, there are 39 *Hox* genes grouped into four ~120 kb clusters on four chromosomes, *HoxA* to *HoxD* in the mouse and *HOXA* to *HOXD* in man (Fig. 1). It is believed that these clusters originated by two duplication events through evolution from the same ancestral cluster that gave rise to the *Drosophila* homeotic genes (Kappen et al., 1989). In *Drosophila* there are 8 homeotic genes split into two clusters, the *bithorax* (BX-C) complex and the *Antennapedia* (ANT-C) complex, both located in tandem on the same chromosome (McGinnis and Krumlauf, 1992). The vertebrate ancestral cluster is believed to resemble what is found in the cephalochordate *Amphioxus*, a single cluster containing 13 *Hox* genes (Garcia-Fernandez and Holland, 1996). This suggests that the duplication events that led to the *Hox* clusters in vertebrates occurred close to the origin of the vertebrate line. In mammals, genes that occupy the same position in the cluster are more closely related than neighboring genes or any other gene in the cluster and are termed **paralogs**. This alignment arranges the mammalian *Hox* genes into 13 such paralogous groups (Krumlauf, 1994). Not all paralogous groups contain 4 genes; some paralogs have been lost following the duplication events. Paralogs in the 3' end of the cluster present similar patterns of expression in the developing embryo suggesting that these gene products perform overlapping functions (Maconochie et al., 1996). Gene targeting studies generating single and compound paralog mutants suggested that the paralogs are mostly redundant in function despite some unique functions (Chen and Capecchi, 1997; Condie and Capecchi, 1994; Davis et al., 1995; Fromental-Ramain et al., 1996a; Fromental-Ramain et al., 1996b; Gavalas et al., 1998; Horan et al., 1995b; Manley and Capecchi,

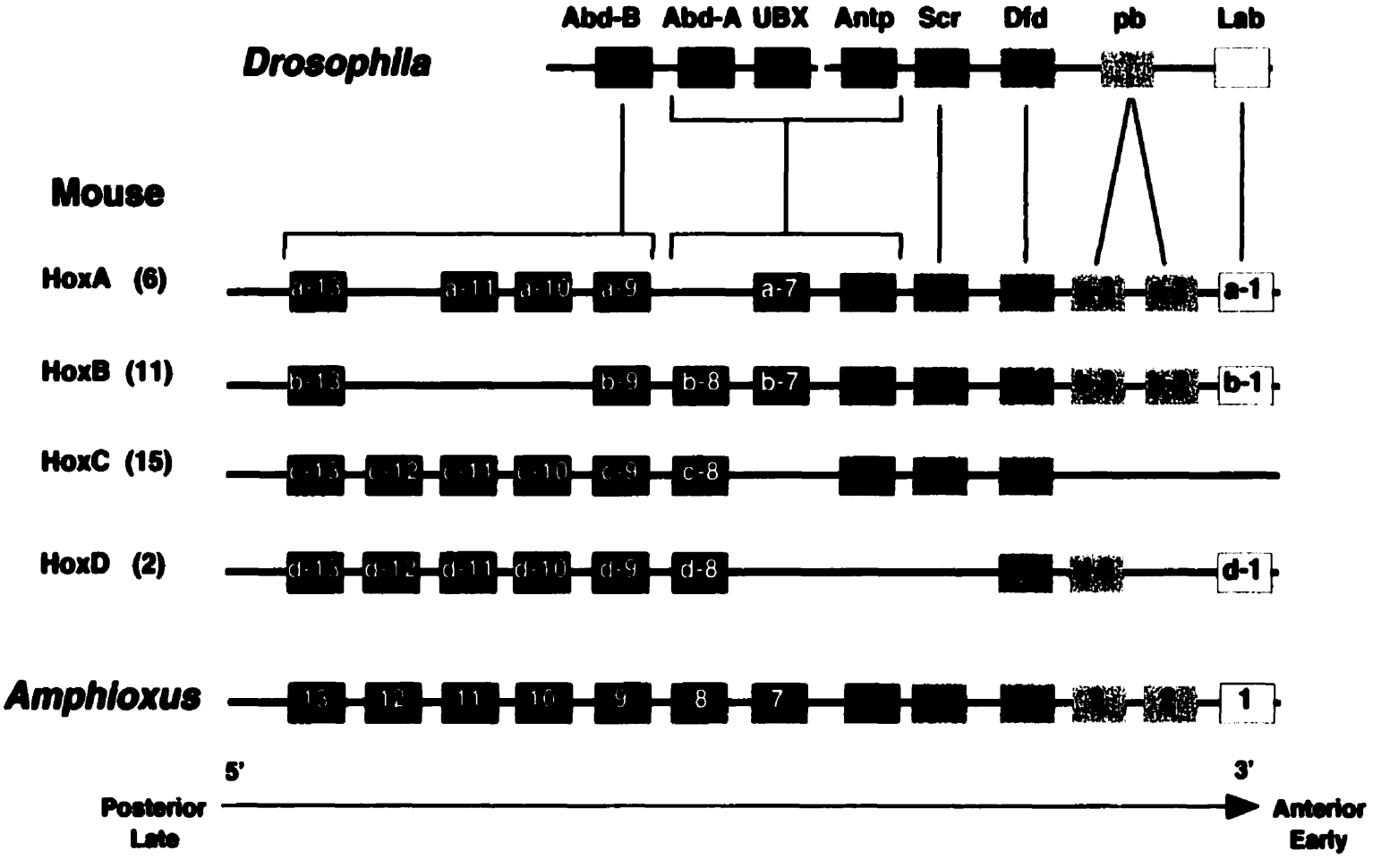
1997; Manley and Capecchi, 1998; Studer et al., 1998; Warot et al., 1997). Another interesting feature of the genomic organization of the mammalian *Hox* genes is that all the genes are transcribed from the same strand (Krumlauf, 1994). Such an observation supports the theory of tandem duplications of *Hox* genes from a proto-*Hox* ancestral gene (Lewis, 1978). This theory predicted that a first duplication resulted in two *Hox* genes: an "anterior" or "head" gene and a "posterior" or "tail" gene that independently functioned in the anterior and posterior body specification, respectively. An unequal crossover subsequently generated a trunk *Hox* gene (Gehring et al., 1994a). Further duplications resulted in five *Hox* genes constituting the last ancestor before the bifurcation of the insect and vertebrate lines (Schubert et al., 1993). Subsequent duplications resulted in the 13 members of the vertebrate ancestral cluster. Data supporting this theory include, for example, the presence of one *Hox* gene in sponge (Finnerty and Martindale, 1998) or two *Hox* genes in Hydra: *cnox1* as the "head" gene and *cnox3* as the "tail" gene (Gauchat et al., 2000; Peterson and Davidson, 2000).

Evolution might have selected for *Hox* gene clustering for various reasons. One, which we know about, is enhancer sharing (Gould et al., 1997; Sharpe et al., 1998). Another, as proposed by a current model, might be the existence of a "global" enhancer at one end of the *Hox* cluster that would exert a regulatory effect on all the *Hox* genes in the cluster, such as a progressive de-heterochromatinisation (Dollé et al., 1989; Kondo et al., 1998; Vanderhoeven et al., 1996), a maintenance or imprinting system (Gaunt and Singh, 1990; Orlando and Paro, 1995; Pirrotta, 1997a), or by analogy to the β globin cluster, a locus control region (Dillon and Grosveld, 1993).

Figure 1: The *Hox* family in mammals and its evolutionary relation to the families in *Drosophila* and *Amphioxus*.

Schematic representation of the *Hox* families. Each box represents a gene. In *Drosophila*, the single *Hox* cluster has been split to either end of chromosome III. In mice, there are 4 clusters, *HoxA* to *HoxD*, on 4 chromosomes (chromosomes 6, 11, 15 and 2) which comprise 39 *Hox* genes that can be aligned in 13 paralog groups. These 13 groups are represented by the ancestral *Hox* cluster of *Amphioxus* (bottom). The color shading represents the relatedness between *Hox* genes. All *Hox* genes, in the exception of *Dfd*, are transcribed in the same orientation, left to right, as diagrammed. Genes that are at the 3' end of the cluster are expressed earlier on and more anteriorly than those at the 5' end of the cluster. *lab, labial; pb, proboscipedia; Dfd, Deformed; Scr, Sex comb reduced; Antp, Antennapedia; Ubx, Ultrabithorax; AbdA, Abdominal A; AbdB, Abdominal B.*

Hox Clusters



In addition to the conservation of the genomic organization of the *Hox* clusters during evolution, the *Hox* coding sequences, expression patterns, interacting partners, functional specificity and, to a lesser extent, regulatory elements have also been conserved. It is believed, however, that divergence in body patterns resulted primarily from changes in the regulatory elements controlling the expression of *Hox* genes rather than from divergence in their coding sequences (Carroll, 1995; Carroll, 2000). For example, the CDX enhancer of the *Hoxc8* gene contains a 2 bp change between the mouse and the chick. Such a divergence led to a shift of the *Hoxc8* anterior boundary in the somitic mesoderm from somite 14 in the mouse to somite 20 in the chick (Belting et al., 1998).

2.2 *Hox* genes functions as revealed from their expression patterns: Insights from gene targeting studies

In the mammalian embryo, the spatial and temporal pattern of expression of the *Hox* genes along the A/P axes is parallel to their chromosomal distribution. This defines **the colinearity rule**. Genes that are at the 3' end of the cluster are expressed earlier on and more anteriorly in the body than genes that are at the 5' end of the cluster (Krumlauf, 1994). During early embryogenesis, the establishment of *Hox* gene expression occurs in two phases: initiation and maintenance (Deschamps and Wijgerde, 1993). For all *Hox* genes, expression initiates at the posterior end of the embryo and progresses more anteriorly until it reaches a well defined limit, the anterior border, which varies generally for different paralogous groups (see below) (Holland and Hogan, 1988). Once the anterior border is reached, the expression is maintained. Therefore, *Hox* genes are

expressed in different but overlapping domains along the A/P axes, with each cell, or group of cells, expressing a special combination of *Hox* genes, **a *Hox* code** (McGinnis and Krumlauf, 1992). The *Hox* code is believed to determine cell fate and lie at the basis of body patterning: different *Hox* codes would instruct different regions of the body to develop into their resulting structures.

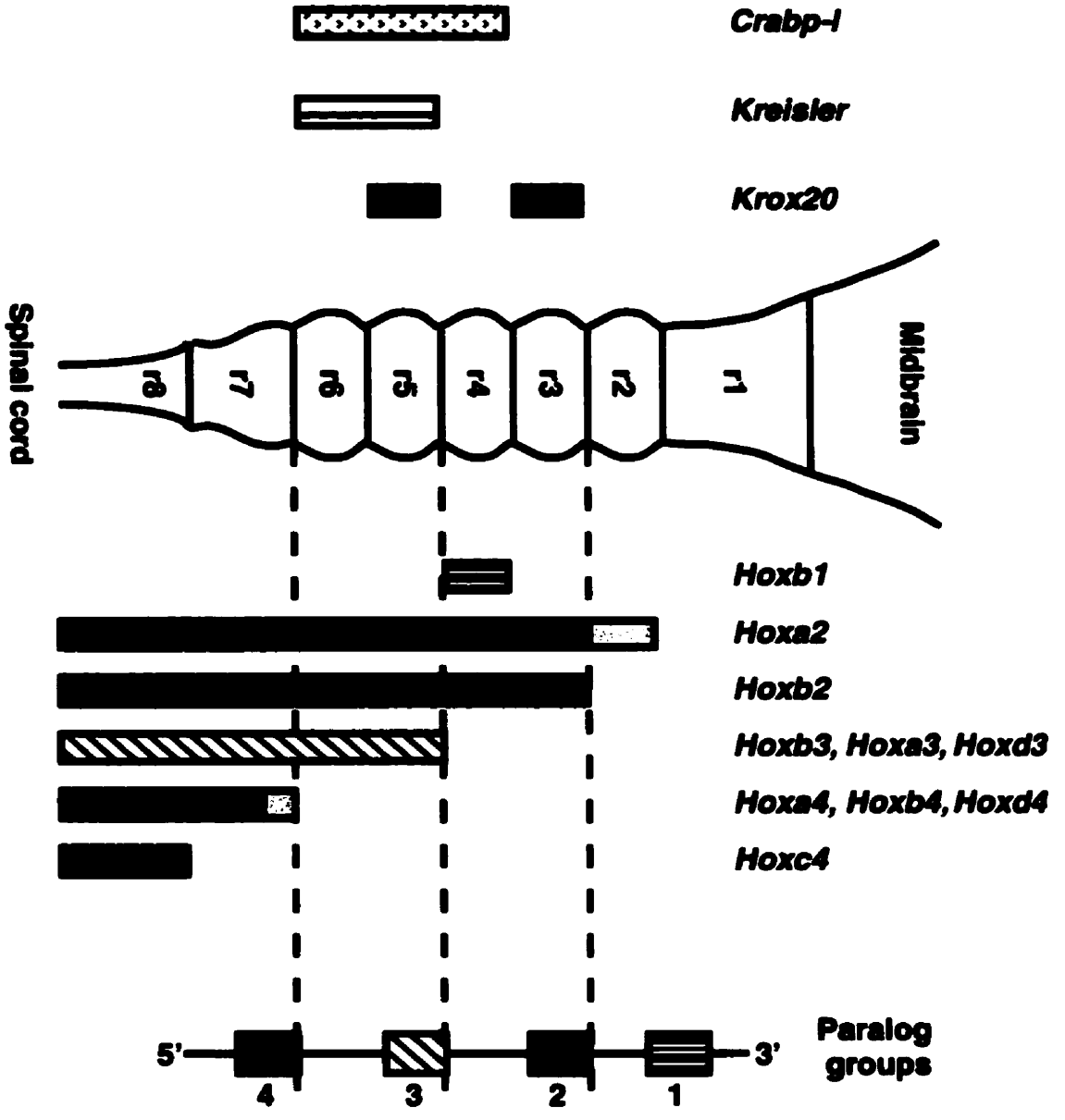
Hox gene expression and function have been studied in greater detail in the CNS, somitic mesoderm and the limb buds. In the CNS, the most anterior border of *Hox* expression is in the hindbrain (Hunt et al., 1991; Murphy et al., 1989; Wilkinson et al., 1989b). No *Hox* expression is detected in either the midbrain or the forebrain. During embryogenesis, the hindbrain is transiently divided into 7 lineage-restricted constrictions called "rhombomeres" (r) (cells within individual rhombomeres are not free to mix with those of neighboring rhombomeres) (Fraser et al., 1990; Lumsden, 1990; Lumsden, 1999). Similarly, the presomitic mesoderm is organized into segments called "somites". The anterior borders of *Hox* gene expression have been shown, by *in situ* hybridization or using *lacZ* reporters in transgenic analysis, to localize to boundaries between rhombomeres in the CNS (for *Hox* genes from groups 1 to 4) (Hunt et al., 1991; Lumsden and Krumlauf, 1996; Murphy et al., 1989; Wilkinson et al., 1989b) and to positions of major morphological transitions such as the vertebrae or limb buds (for *Hox* from paralogous groups 5 to 13) (Burke et al., 1995). In the CNS, the most anteriorly expressed *Hox* gene is *Hoxa2* reaching r1/2 boundary. *Hoxb2* follows with an anterior border up to r2/3 boundary (Wilkinson, 1993). Group 1 *Hox* genes are the first to be expressed in the embryo at around 7.5-7.75 days post coitum (dpc), however they defy the spatial colinearity rule. Both *Hoxa1* and *Hoxb1* are expressed initially with an anterior

border at the presumptive r3/4 boundary, thus more posterior than group 2. At 8.5 dpc, the expression of both genes retracts caudally and is downregulated except in r4 where *Hoxb1* expression stays strong until 12.5 dpc (Murphy and Hill, 1991). With the exception of *Hoxa2*, *Hox* genes from paralogous groups 2 to 4 follow perfectly the colinearity rule. In addition, their expression pattern is staggered by a two-rhombomere interval (Wilkinson, 1993). As mentioned above, *Hoxb2* is expressed up to r2/3 boundary. Group 3 gene expression extends anteriorly up to the r4/5 boundary and group 4 up to the r6/7 boundary (Fig. 2). In addition to being expressed in the hindbrain, *Hox* genes from paralogous groups 1 to 4 are also expressed in the neural crest cells (NCC) that derive from the rhombomeres and migrate to their respective branchial arches.

The expression patterns of *Hox* genes reflect some of their functions. Gene targeting studies of most *Hox* genes have been conducted. In addition, double and compound mutants were obtained in some instances. Mutations in *Hox* from groups 1 to 4 revealed that the function of these gene products is most important in their anterior segment of expression and in the NCC that derive from it. *Hox* from groups 1 to 3 are thus important for the development of the hindbrain and the NCC-derived structures. Mutations of *Hoxa1* and *Hoxb1*, for instance, resulted in defects in the hindbrain where rhombomeres were greatly reduced or missing. In addition, defects were observed at the level of the branchial arches, such as the malformation or absence of the pharyngeal arch-derived acoustic structures (styloid bone, stapes, hyoid bone, tympanic ring), and at the level of the migration of r4-derived cranial motor neurons, leading to facial paralysis [Lufkin, 1991 #211; Chisaka, 1992 #2902; Carpenter, 1993 #739; Dollé, 1993 #2979; Mark, 1993 #3953; Goddard, 1996 #3954; Studer, 1996 #3955; Gavalas, 1998 #3045;

Figure 2: The expression patterns of *Hox* genes, *Crabp I*, *Krox20* and *Kreisler* in the mouse hindbrain.

Schematic representation of murine *Hox* gene expression in the hindbrain at 9.5 dpc. The expression pattern of some genes involved in the regulation of *Hox* expression is also depicted (*Crabp I*, *Krox20* and *Kreisler* genes). At this stage of development, the hindbrain is divided into 8 A/P segments termed rhombomeres (r1-r8). *Hox* genes from paralog groups 1 to 4 are represented by a generalized cluster with 4 boxes of different shadings, each representing a gene. Gene expression domains are represented by vertical bars with shading corresponding to the respective paralog group. *Hoxb1*, the only paralog group 1 gene expressed at this stage, is restricted to r4. The anterior border of expression of genes from groups 2, 3 and 4 follow the two-rhombomere interval rule with the exception of *Hoxa2* that extends more anteriorly up to r1/r2 boundary. *Krox20* expression is restricted to r3 and r5 and regulates *Hoxa2* and *Hoxb2* in these rhombomeres. *Kreisler* is expressed in r5 and r6 and its product KRML1 has been shown to regulate the expression of *Hoxa3* and *Hoxb3* in these rhombomeres. *Crabp I* is the cytoplasmic retinoic acid (RA)-binding protein; its segmental expression pattern in the hindbrain in r4-r6 is believed to regulate the levels of RA in these cells to modulate the RA-regulation of *Hox* gene expression.


Crabp-I
Kreisler
Krox20
Spinal cord
r8
r7
r6
r5
r4
r3
r2
r1
Midbrain
Hoxb1
Hoxa2
Hoxb2
Hoxb3, Hoxa3, Hoxd3
Hoxa4, Hoxb4, Hoxd4
Hoxc4
5'
3'
4
3
2
1
Paralog groups

Studer, 1998 #3684]. Starting from paralogous group 3, the mutants showed homeotic transformations at the level of their vertebrae. For example, mutation of *Hoxd4* resulted in vertebral malformations and homeotic transformations in the cervical region; the cervical vertebra 2 (C2) taking the identity of that of cervical vertebra 1 (C1) (C2 to C1 transformation). This resulted in an ectopic anterior arch at C2, malformed basioccipital bone and abnormal neural arches at C1, C2 and C3 (Horan et al., 1995a). Interestingly, a compound mutant of Hoxa4/b4/d4 revealed that these genes are partially redundant in their function: The mutant presented an increased number of vertebrae transformed into C1 identity, such as an ectopic anterior arch formed from C2 to C5 (Horan et al., 1995b). This dosage-dependant effect suggests that multiple *Hox* genes may function synergistically towards the development of one specific structure. In addition to skeletal phenotypes, defects in other systems were also observed. For example, the *Hoxa5* mutant presented defects in the respiratory system as well as in the gastro-intestinal tract (Aubin et al., 1998; Aubin et al., 1997; Jeannotte et al., 1993). These phenotypes reflect the normal expression pattern of this *Hox* gene and its function in these organs. Most other *Hox* genes are also expressed in the gut. However, it is difficult to describe the expression pattern of *Hox* genes in this organ with respect to the colinearity rule because of the complexity of its cell origins (Beck et al., 2000). More 5' *Hox* genes, starting from paralogous group 9 (*AbdB*-related *Hox* genes), besides being expressed along the main A/P axis are also expressed within nested domains of the developing limb buds. Genes from the *HoxA* and *HoxD* clusters respect the spatio-temporal colinearity rule, in that *Hoxa9* and *Hoxd9* are expressed earlier and more anteriorly than more 5' genes. *Hoxa9* and *Hoxd9* are expressed in almost the whole limb bud while more 5' genes are activated

sequentially in restricted more posterior and distal domains (Dollé et al., 1989; Haack and Gruss, 1993). The *HoxC* genes present a different pattern of expression in the limb. More 3' genes are expressed in the forelimb, more 5' genes are expressed in the hindlimb and intermediate gene products are present in both fore- and hindlimbs (Nelson et al., 1996; Peterson et al., 1994). Within the limb, *HoxC* genes are expressed anteriorly and proximally (Nelson et al., 1996). The limb is divided into three segments: the antero-proximal domain develops into the upper limb (stylopod), the central domain into the lower limb (zeugopod) and the postero-distal domain into the hand/foot (autopod). Defects in the limbs are observed in mutant *Hox* genes from paralogous groups 9 to 13, as expected from their pattern of expression. Group 9 is essential for the upper limb (Fromental-Ramain et al., 1996a), group 11 for the lower limb (Davis and Capecchi, 1994; Davis et al., 1995; Favier et al., 1995; Favier et al., 1996; Small and Potter, 1993) and group 13 for the hand/foot (Davis and Capecchi, 1996; Dollé et al., 1993; Fromental-Ramain et al., 1996b). Groups 8 and 10 are required for the proper development of the spinal nerves that innervate the limbs as mutations in members of these paralogous groups resulted in impaired limb mobility (Carpenter et al., 1997; Favier et al., 1996; Le Mouellic et al., 1992; Rijli et al., 1995; Tiret et al., 1998).

In addition to presenting defects in the limb, mutants from groups 9 to 13 showed skeletal malformations and transformations in the thoracic, lumbar and sacral regions, defects in the hematopoietic system (*Hoxa9*) (Lawrence et al., 1997) and defects in the urogenital system (groups 10 and 11) (Benson et al., 1996; Davis et al., 1995; Hsieh-Li et al., 1995; Satokata et al., 1995) and at the end of the digestive tract (groups 12 and 13) (Kondo et al., 1996; Podlasek et al., 1997; Warot et al., 1997). In summary, the

expression pattern of *Hox* genes, and consequently the defects observed in their mutants, respect once again the colinearity rule. Deletion of the most 3' genes revealed defects in the hindbrain while defects of the urogenital system and the end of the digestive tract were observed in the mutants of the most 5' genes.

2.3 Regulation of *Hox* gene expression

Throughout evolution, the regulatory mechanisms controlling *Hox* gene expression have greatly diverged between species. In *Drosophila*, for instance, *Hox* genes are under the control of a genetic regulatory hierarchy composed solely of transcription factors. The hierarchy is headed by the maternal effect genes such as *bicoid*, *nanos* and *caudal* that establish the A/P axis of the embryo. During oogenesis, the mRNAs from these genes are transcribed and localized to the anterior (*bicoid*) and posterior (*nanos* and *caudal*) ends of the egg. After fertilization, the mRNAs are translated and the respective proteins form concentration gradients at the opposite poles of the embryo. These proteins function as morphogens. They regulate downstream genes with differential sensitivity to their concentration gradients. Downstream of the maternal effect genes are the segmentation genes that include the gap genes, the pair-rule genes and the segment polarity genes. These genes are responsible for the final subdivision of the *Drosophila* body into repetitive segments. The identity of these segments is finally contributed by the action of the homeotic genes or *Hox* genes that come under the control of the segmentation genes. In addition, some *Hox* genes have also been shown to be autoregulated (regulated by their own products) or crossregulated (by the products of other *Hox* genes).

The upstream regulators described above constitute one level of *Hox* regulation. A second more global level involves the modulation of the higher order chromatin structure around the *Hox* genes. Proteins from the polycomb group (PcG) or the trithorax group (trxG) affect the chromatin structure to maintain the expression of *Hox* genes in either a repressed or an activated state, respectively (Akam, 1987; Ingham, 1988).

In *Drosophila*, the initial activation of *Hox* gene expression precedes cellularization of the blastoderm embryo and hence its regulation is simply associated with the cascade of interactions between the transcriptional regulators described above. In mammals, the expression of *Hox* genes begins during gastrulation when cells are dividing, migrating and responding to signaling cues from both their environment and the surrounding cells (Akam, 1989; Ingham, 1988). The situation in mammals is hence more complex and requires, in addition to transcriptional regulators, inputs from signaling molecules (Krumlauf, 1994). Mutational and deletional analysis in transgenic mice, in combination with genetic studies, functional studies in tissue culture and *in vitro* DNA-binding studies have been successful in identifying some of the regulators of mammalian *Hox* gene expression. The upstream regulators identified to date include the retinoic acid receptors (RXR•RAR complexes), HOX•PBX•MEIS complexes (involved in *Hox* genes auto- and crossregulation), KROX20, KRML1, CDX, AP2 and GATA1 family members (Fig. 3). Interestingly, some of these genes including the retinoic acid receptors, cellular retinoic acid binding proteins *(CRABP I)*, *Krox20* and *Kreisler* present a segmentally-restricted pattern of expression in the developing hindbrain that suggest a role in patterning through *Hox* regulation (Fig. 2). PcG group and trxG group members are also implicated in the maintenance of mammalian *Hox* gene expression, as it is the case in

Drosophila (Akam, 1987; Ingham, 1988; McGinnis and Krumlauf, 1992). In the following subsections, I will describe the upstream regulators listed above and their corresponding *cis*-acting elements characterized in the mammalian *Hox* genes. For each regulatory mechanism, I will detail only one or two representative studies in which the enhancer has been identified and shown to be functional *in vivo* to direct the correct pattern of expression of the respective *Hox* gene.

2.3.1 *Regulation by RXR•RAR complexes*

Retinoids are active metabolites of vitamin A and are the transducers of its pleiotropic effects in higher vertebrates. They are involved in many aspects of embryogenesis, one of which, very early in development, is the regulation of some of the *Hox* genes (for review, see Means and Gudas, 1995). A critical level of retinoids is maintained in the body. Deficiency leads to various defects during embryogenesis (vitamin A deficiency syndrome) and cancers in adult, while excess has potent teratogenic effects on mammalian embryos (Means and Gudas, 1995). It is believed that these defects are partly caused by misregulation of *Hox* gene expression. Among different studies supporting this hypothesis, Zhang *et al.* showed that misexpression of *Hoxa1* in the mouse partially reproduced the RA-induced phenotypes in the hindbrain and NCC-derived structures (Zhang et al., 1994).

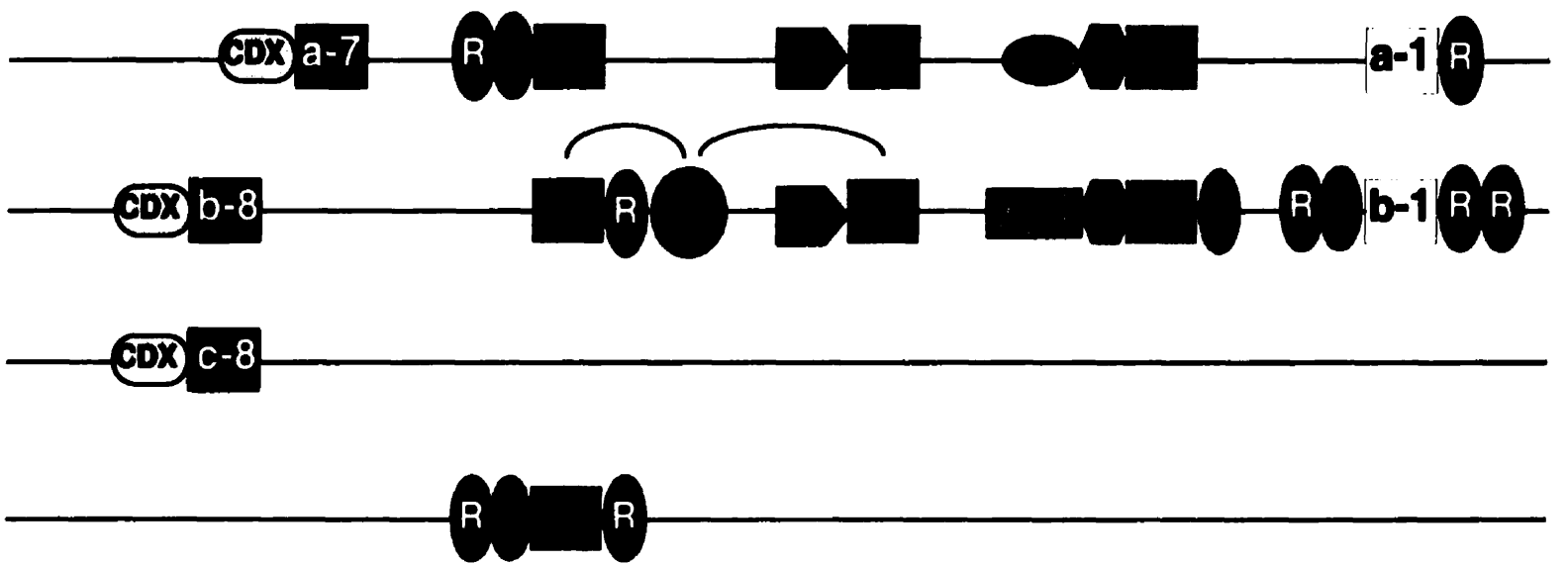
Many studies have focused on understanding how retinoids transduce their multiple effects. A breakthrough came in 1987 when the first receptor for retinoic acid (RAR) was discovered (for review, see Giguère, 1994). These receptors, members of the nuclear receptor superfamily, are ligand-inducible transcription factors. To date there are

two groups of retinoid receptors, the retinoic acid receptors (RAR α, β, and γ), that respond to both all-*trans* and 9-*cis* retinoic acid, and the retinoid X receptors (RXR α, β, and γ) that are activated by only 9-*cis* retinoic acid. RARs and RXRs function as a RXR•RAR heterodimeric complex to transduce the effects of RA and regulate downstream targets. In the absence of ligand, RXR•RAR repress transcription by the recruitment of a corepressor complex consisting of the nuclear receptor corepressor (N-coR) or the related corepressor (SMRT), mSIN3A/mSIN3B (the mammalian homologues of the yeast global repressor SIN3) and members of the histone deacetylase (HDAC) family. Upon ligand binding, a conformational change is induced in the receptor ligand-binding pocket leading to dissociation of the corepressor complex and subsequent association of a coactivator complex with histone acetyl transferase (HAT) activity (Glass and Rosenfeld, 2000). RXR•RAR recognize a DNA consensus sequence termed the RARE (retinoic acid receptors response element). The RARE consists of a direct repeat of the sequence (A/G G G/T TCA) separated by either 2 (DR2) or 5 bp (DR5) (Mangelsdorf et al., 1994).

RAREs of both types have been identified in regulatory regions of some *Hox* genes and have been shown by transactivation assays in tissue culture systems, transgenic analyses and targeted mutation of the enhancer to be essential in the regulation of these *Hox* genes. DR5-type RAREs are found in the 5' regulatory regions of *Hoxa4* (Packer et al., 1998) and *Hoxd4* (Moroni et al., 1993; Pöpperl and Featherstone, 1993) and in the 3' regulatory regions of *Hoxa1* (Dupé et al., 1997; Frasch et al., 1995; Langston and Gudas, 1992), *Hoxb1* (Huang et al., 1998; Langston et al., 1997), *Hoxb4* (Gould et al., 1998) and *Hoxd4* (Morrison et al., 1997; Zhang et al., 2000).

Figure 3: Transcriptional regulators of *Hox* gene expression.

Schematic representation of *Hox* genes with characterized *cis*-regulatory elements. These include *Hox* genes from paralog groups 1 to 4 and *Hox* genes from groups 7 and 8. *Cis*-regulatory elements are represented in colored circles or boxes. "R", RARE; A, autoregulatory element; C, crossregulatory element; A/C, shared enhancer used for autoregulation of *Hoxb4* and crossregulation of *Hoxb3*. For A, C and A/C, the transcription factors in play are HOX, PBX and MEIS/PREP1 proteins. K, Krox20 binding site; KR, Kreisler/KRML1 binding site.


CDX
a-7
R
a-1
R
CDX
b-8
R
R
b-1
R
R
CDX
c-8
R
R

To date, DR2-type RAREs have been described in only one *Hox* gene, *Hoxb1*. *Hoxb1* possesses two such RAREs, one in its 5' enhancer and the second in its 3' enhancer (Marshall et al., 1994; Studer et al., 1994). Thus, in total, *Hoxb1* is regulated by three RAREs. Each RARE has been shown to mediate only some aspects of the *Hoxb1* expression pattern in the developing embryo. The 3' DR2 RARE is essential for the early expression pattern of *Hoxb1* (at 7.75 dpc) but not for its late expression in r4 (Marshall et al., 1994). The 5' DR2 RARE is required to repress the expression of *Hoxb1* from r3 and r5, thus restricting its expression domain to r4 (Studer et al., 1994), and the 3' DR5 RARE functions in the regulation of *Hoxb1* expression in the gut (Huang et al., 1998; Langston et al., 1997). The combinatorial information from all three RAREs, along with contributions from other enhancers such as the *Hoxb1* autoregulatory element (ARE) (see below), would reconstitute the correct pattern of expression of *Hoxb1* during development.

The characterization of RAREs in *Hox* gene enhancers is in perfect agreement with previously reported studies on the effects of RA on *Hox* expression. The first evidence of regulation by RA came from studies in embryonal carcinoma (EC) cells showing sequential induction of the *HOXB* genes in response to RA in a 3' to 5' direction (Breier et al., 1986; Deschamps et al., 1987; Mavilio et al., 1988; Simeone et al., 1990). Subsequently, *Hox* genes were shown to be induced by RA *in vivo*: in the limb bud, prevertebrae and the neural tube (Conlon and Rossant, 1992; Kessel, 1992; Kessel and Gruss, 1991; Oliver et al., 1990). Recently, a study analyzing the disruption of retinaldehyde dehydrogenase-2 gene (Raldh2), encoding an enzyme essential in RA biosynthesis, showed severe defects in early embryogenesis. Examination of *Hox* gene

expression in this mutant background revealed altered expression of various *Hox* genes including *Hoxa1*, *b1*, *a3*, *b3*, *a4*, *b4* and *d4* (Niederreither et al., 1999; Niederreither et al., 2000). These data indicate that RA controls the above mentioned genes either directly or indirectly.

2.3.2 *Auto- and cross regulation*

Direct regulation of *Hox* genes by their own products (autoregulation) or by the products of other *Hox* genes (crossregulation) has been discovered first in *Drosophila*, and shown to provide a means of cross-talk among *Hox* genes (McGinnis and Krumlauf, 1992). This mode of regulation has been conserved in mammals, suggesting that regulation of *Hox* by HOX (also true for CDX) is an ancient strategy while that by other upstream factors, such as RXR•RAR, KROX20 and KRML1, is an innovation. Auto- and crossregulatory elements have been described in a number of mammalian *Hox* genes including *Hoxb1* (Barrow et al., 2000; Pöpperl et al., 1995; Studer et al., 1998; Zhang et al., 1994), *Hoxb2* (Ferretti et al., 2000; Jacobs et al., 1999; Maconochie et al., 1997), *Hoxb3* (Gould et al., 1997), *Hoxa4* (Packer et al., 1998), *Hoxb4* (Gould et al., 1997) and *Hoxd4* (Pöpperl and Featherstone, 1992).

Hoxb1 is autoregulated (Pöpperl et al., 1995) and is crossregulated by HOXA1 (Studer et al., 1998; Zhang et al., 1994). The *Hoxb1* autoregulatory element (ARE) is a 120 bp enhancer that includes three binding sites for HOX•PBX complexes and a conserved block (block 1) with a MEIS/PREP1-like binding site. It is sufficient to direct reporter expression to r4 in transgenic analysis, is active in *Drosophila* only in the presence of LABIAL (*Drosophila* homologue of HOXB1) and EXD (HOX cofactor,

Drosophila homologue of PBX) and is specifically bound by a HOXB1•EXD complex in electromobility shift analysis (EMSA) (Pöpperl et al., 1995). Recent evidence suggests a third player in the HOX•PBX complex on the *Hoxb1* (ARE). This involves members of the MEIS/PREP1 family, where MEIS1 and PREP1 have been shown to interact with HOXB1 and PBX in a trimeric complex (Berthelsen et al., 1998a; Jacobs et al., 1999) (see below). One recent report revealed that a MEIS/PREP1-like binding site (block1) within the *Hoxb1* ARE is not required to direct *Hoxb1* autoregulation in r4, and suggested that the formation of the trimer on this element is not essential for its activity (Ferretti et al., 2000). However, this study did not address, the possibility of the formation of a HOX•PBX•MEIS/PREP1 trimer in which MEIS/PREP1 function in a DNA-binding independent manner. In addition, to rule out that MEIS/PREP1 binding is not required, a more extensive mutagenesis of all possible MEIS/PREP1 binding sites within the *Hoxb1* ARE needs to be conducted, followed by the examination of the mutant transgene expression in r4.

Evidence for HOXA1 crossregulation came from different studies. First, ectopic expression of HOXA1 in anterior regions during early development results in misexpression of *Hoxb1*, but not *Hoxd1*, in neuroepithelial cells of r2 (Zhang et al., 1994). Second, the presence of HOXA1 in a *Hoxb1* null mutant compensates for the absence of HOXB1 and maintains the correct expression of a *Hoxb1-lacZ* transgene (Studer et al., 1998). In contrast, in *Hoxa1* null embryos, *Hoxb1* expression is only initiated in r4 but at weaker levels. Recent evidence suggests that the precise function of HOXA1 in the regulatory loop of *Hoxb1* is to set its correct expression in the anterior region of r4 (Barrow et al., 2000). In summary, *Hoxb1* expression is controlled by a

combinatorial regulatory mechanism involving 3 RAREs and an ARE. One can speculate that a hierarchy of regulatory loops act sequentially to direct the correct expression pattern of *Hoxb1* and perhaps of other *Hox* genes that possess both RAREs and AREs (as in the case of *Hoxa4*, *Hoxb4* or *Hoxd4*). First, RA directly stimulates early *Hox* expression, restricts its expression domains to specific segments and indirectly stimulates autoregulatory loops. HOX proteins then take over and mediate their action through AREs to induce or maintain later expression patterns.

Another example of crossregulation by HOXA1 and HOXB1 is the regulation of the *Hoxb2* r4 enhancer. This enhancer is responsive to *Hoxa1*, *Hoxb1* and *Drosophila Lab* ectopic expression in transgenic mice but not to that of *Hoxb2* or *Hoxb4*. In *Drosophila*, the enhancer is active only in the presence of LAB and EXD suggesting an evolutionary conserved role of LAB-related proteins in the regulation of *Hoxb2* expression. Similar to the regulation of the *Hoxb1* ARE by HOX•PBX and possibly MEIS/PREP1 in a trimeric complex, the *Hoxb2* r4 enhancer has been recently shown to require the formation of such a trimeric complex for full activity (Ferretti et al., 2000; Jacobs et al., 1999).

Other *in vivo* evidence suggesting the regulation of *Hoxb2* r4 expression by HOXB1 came from the observation that *Hoxb2* is no longer upregulated in r4 in *Hoxb1* null mutants (Maconochie et al., 1997). Since HOXB1, but not HOXA1 or HOXD1, is the only LAB-related protein that is present in r4 at the stage of *Hoxb2* upregulation, it is likely the endogenous regulator of *Hoxb2 in vivo*.

Auto and cross-regulation are essential in the segmentation and patterning of the hindbrain as best exemplified by the recent study analyzing *Hoxa1/Hoxa2* double null

mutants (Barrow et al., 2000). This study elegantly showed that, in the absence of HOXA1 and HOXA2, the hindbrain is completely smooth and devoid of any rhombomere boundaries at all stages examined. Initially, HOXA1 establishes the correct expression of *Hoxb1* in the anterior portion of r4. Following the regression of *Hoxa1* and *Hoxb1* expression from the hindbrain, *Hoxb1* expression in r4 becomes solely maintained by its autoregulatory loop. This restricted expression in r4, but not in more posterior rhombomeres, is mediated by the repressive action of *Kreisler*, which is itself under the control of HOXA1 in r5 (see below). Similarly, *Krox20* initiation in r3 is under signaling from *Hoxa1* and *Hoxb1* derived from r4 while its proper expression and expansion in r3 is regulated by the products of *Hoxa2* and perhaps *Hoxb2*, two of its downstream targets. In addition, as mentioned above, the expression of *Hoxb2* in r4 is crossregulated by HOXB1 and HOXA1. Therefore, the absence of both HOXA1 and HOXA2 leads to misregulation of various genes whose expression patterns distinguish the different rhombomeres, resulting in loss of rhombomere identity. This example illustrates well the cross-talk among *Hox* genes and the roles of other upstream regulators such as KROX20 and KREISLER in the patterning of the hindbrain.

2.3.3 *KROX20*

Krox20, also known as *Egr2* (early growth response gene 2), was originally cloned from mouse fibroblast cells as an immediate-early gene that is quickly induced at the G0/G1 transition of the cell cycle, in response to serum stimulation, or to mitogens (Chavrier et al., 1988; Joseph et al., 1988). It encodes a transcription factor with three tandem C2-H2 zinc fingers as its DNA-binding domain. During early development, *Krox*

20 expression pattern is restricted to r3 and r5 in the hindbrain and to early NCC. Subsequently, its expression decays and becomes detectable in specific hindbrain nuclei, NCC-derived boundary caps, and glial components of the cranial and spinal ganglia (Wilkinson et al., 1989a). Targeted mutation of *Krox20* resulted in defects in hindbrain development with marked reduction or loss of r3 and r5. One consequence of rhombomere loss and hindbrain defects is the disorganization of cranial nerves as they enter the brain stem (Schneider-Maunoury et al., 1997; Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993). Together with its restricted expression pattern, the phenotypes of *Krox20* null mutants indicated a role in specifying segment identity, possibly through regulation of *Hox* genes. The first evidence of *Hox* regulation by KROX20 was revealed in the case of *Hoxb2* (Sham et al., 1993). Deletion analysis of the *Hoxb2* upstream region mapped an r3/5 enhancer in transgenic mice that contains three KROX20 binding sites. Mutation of all three sites together or of only the first site with the highest affinity to KROX20 abolished transgene expression in r3 and r5. This suggested a role for KROX20 in the direct regulation of *Hoxb2* in these rhombomeres. However, KROX20 binding sites, when multimerized, were not sufficient to direct the correct expression of *Hoxb2* to r3/5 (Sham et al., 1993). An additional *cis*-acting element, box 1, conserved in the mouse and chicken enhancers was also required. Box1 and KROX20 binding sites can act as an r3/5 enhancer when oligomerized (Nonchev et al., 1996a; Vesque et al., 1996). The regulation of *Hoxb2* by KROX20 directed the analysis of whether *Hoxa2*, the only known paralog of *Hoxb2*, was also downstream of KROX20. The first evidence of such a regulation came from the observation that *Hoxa2* expression was clearly lost in r3 in *Krox20* null mutants. Transgenic analysis followed and mapped

an 809 bp r3/r5 enhancer containing two KROX20 cooperative binding sites. This enhancer was active in r3 and r5, NCC of branchial arch 2, dorsal root ganglia, somatic mesoderm and lateral plate mesoderm. Mutation of the two KROX20 binding sites abolished expression in r3 and r5 but did not affect the other expression domains. Thus, similarly to *Hoxb2*, these sites are required but not sufficient for the r3/5 restricted pattern of *Hoxa2* expression. The requirement of an additional *trans*-acting factor for correct r3/5 expression suggests that KROX20 might cooperate with different factors to direct expression in different tissues (Nonchev et al., 1996b). Ectopic expression of *Krox20* in either r4 or r6/7 directed *Hoxa2-LacZ* expression to these respective segments in the hindbrain, providing an elegant proof that *Hoxa2* is an *in vivo* target of KROX20 (Nonchev et al., 1996b).

In addition to *Hoxb2* and *Hoxa2*, *Hoxb3* seems to be regulated by KROX20. The normal elevated expression level of *Hoxb3* in r5 is not observed in *Krox20* null mice (Seitanidou et al., 1997). No direct binding of KROX20 to *Hoxb3* regulatory regions was reported however. Moreover, if KROX20 is a regulator of *Hoxb3*, repression mechanisms must be operating in r3 that need to be identified.

Besides regulating *Hox* gene expression, KROX20 has also been shown to act on other genes essential in hindbrain patterning. For instance, KROX20 activates, in r3 and r5, the receptor tyrosine kinase gene *Sek-1* that functions in the segregation of odd-numbered rhombomeres (Mellitzer et al., 1999; Xu et al., 1995; Xu et al., 1999), and represses the *follistatin* gene that is normally expressed in even-numbered rhombomeres (Albano et al., 1994; Feijen et al., 1994). In addition, a more recent report presented evidence for KROX20 regulating the expression of its own antagonists, *Nab1* and *Nab2*,

that function in a negative feedback loop to repress KROX20 transcriptional activation (Mechta-Grigoriou et al., 2000).

Finally, to add to the complexity of the regulation by KROX20 is the implication of RA (Dupé et al., 1999; Gale et al., 1999; Grapin-Botton et al., 1998) and FGF signaling (Marin and Charnay, 2000) in the regulation of its gene expression (as well as that of *Kreisler*, see below).

2.3.4 *KRML1*

KRML1, the product of the *Kreisler* gene, is a basic domain-leucine zipper transcription factor of the Maf subfamily (Cordes and Barsh, 1994). During embryogenesis, its expression is restricted to r5 and r6 in the hindbrain and to the NCC that derive from these two rhombomeres (Cordes and Barsh, 1994; Eichmann et al., 1997). Such an expression pattern is in accordance with the phenotypes of the *Kreisler* mutant mouse (Deol, 1964). The *Kreisler* mutation was generated by X-ray mutagenesis experiments and resulted in a hyperactive mouse running in circles, a behavior caused by defects in the hindbrain and the inner ear. Detailed analysis of *Kreisler* expression revealed however that the *Kreisler* phenotype does not represent a null mutation (Eichmann et al., 1997). *Kreisler* is expressed not only in the hindbrain but also in differentiating neurons of the spinal cord and brain stem, the mesonephros, the perichondrium and the hematopoietic system. The expression of multiple *Hox* genes is affected in the *Kreisler* homozygous mutant including that of *Hoxb1*, *Hoxa3*, *Hoxb3*, *Hoxb4* and *Hoxd4*. In addition, *Krox20*, *Fgf3* and *Crabp1* are not correctly expressed in the hindbrain. Caudal to the r3/4 boundary, the hindbrain is smooth and morphologically

unsegmented (Frohman et al., 1993; McKay et al., 1994). Further investigation of the presence of r5 and r6 using molecular markers indicated the loss of r5 (transformation of r5 into r4 identity followed by apoptosis in r4 to regulate its size) but not that of r6 (Manzanares et al., 1999b; McKay et al., 1994).

The disturbed expression patterns of *Hox* genes in *Kreisler* mutants implies a regulatory function of KRML1 in hindbrain patterning, however it does not indicate a direct role in the regulation of *Hox* genes expression. For this purpose, transgenic analyses were used and revealed *Hoxa3* and *Hoxb3* as direct *in vivo* targets of KRML1 (Manzanares et al., 1999a; Manzanares et al., 1997). The expression of *Hoxa3* and *Hoxb3* extends from the posterior spinal cord up to the r4/5 boundary and is upregulated specifically in r5 and to a lesser extent in r6. In both cases, an r5/6 enhancer was mapped in the upstream regions of the genes and was found to contain KRML1 binding sites. These sites were both required and sufficient to direct the correct expression patterns of the transgene in r5 and r6. In addition ectopic expression of *Kreisler* in other rhombomeres directed the expression of the transgenes to the respective segments. These results strongly implicate KRML1 in *Hox* gene regulation.

2.3.5 *CDX (Caudal)*

Cdx genes are homeobox-containing genes dispersed in the genome. The *Drosophila* homologue of *Cdx*, *Caudal*, is expressed first maternally and later in the zygote in a concentration gradient (with maximum levels at the posterior end of the embryo) as a posterior determinant. Loss of *Caudal* leads to deletion of posterior

structures (Macdonald and Struhl, 1986) and its ectopic expression to abnormal head development and segmentation (Mlodzik et al., 1990).

Homologues of *Cdx* are present in different species including Zebrafish, *Xenopus laevis*, *C. elegans*, chicken, mouse and man (Freund et al., 1998 and references therein). In the mouse, there are three *Cdx* homologues: *Cdx1*, *Cdx2/3* and *Cdx4*. Murine *Cdx* genes are expressed in posterior to anterior concentration gradients along the A/P body axes, in domains overlapping with that of *Hox* gene expression. *Cdx1* and *Cdx2* null mutants show homeotic transformations at the level of the cervical and thoracic vertebrae, phenotypes observed in some *Hox* mutants (Chawengsaksophak et al., 1997; Subramanian et al., 1995). Current data demonstrate that CDX proteins directly regulate the expression of *Hoxb8* (Charité et al., 1998), *Hoxc8* (Belting et al., 1998; Charité et al., 1998; Shashikant and Ruddle, 1996) and *Hoxa7* (Knittel et al., 1995; Min et al., 1996; Min et al., 1998). Regulation of *Hoxa9* by CDX is also suggested (Lorentz et al., 1997) but needs further investigation. These findings suggest that, in contrast to the *Drosophila* CAUDAL, vertebrate CDX transduces positional information by directly regulating *Hox* genes through CDX-binding sites in *Hox* gene position-sensitive enhancers. Recently, *Cdx* genes have been suggested to function as intermediaries that transduce the effects of RA on *Hox* transcription (Houle et al., 2000). *Cdx1* was shown to be regulated by RA *in vivo* and to be a direct target gene for the retinoic acid receptors both *in vivo* and *in vitro*. These findings suggest that for the retinoid-sensitive *Hox* genes that have not been demonstrated to be direct RA targets, other intermediary factors such as the CDX proteins could be in play.

2.3.6 *AP2 family members*

AP2 proteins are transcription factors with no distinct DNA-binding motif (Williams et al., 1988). They bind as dimers through a dimerisation interface composed of 2 α-helices spaced by a span or a linker of 92 aa thus its name helix-span-helix (HSH). Both the HSH and a basic region in the protein are required for DNA-binding (Williams and Tjian, 1991). Two related genes have been cloned as well, *Ap2β* and *Ap2.2* (Chazaud et al., 1996; Moser et al., 1995). The restricted expression patterns of the AP2 family members suggested that they play a developmentally important role. AP2 members are expressed in the NCC and their derived structures. In addition, they are expressed in surface ectoderm, limb bud mesenchyme and in nephric tissues (Chazaud et al., 1996; Mitchell et al., 1991; Moser et al., 1995; Moser et al., 1997; Oulad-Abdelghani et al., 1996). Targeted disruption of *Ap2* resulted in defects in cranial NCC-derived structures, similar to the phenotypes observed in *Hoxa2* null mutants (Gendron-Maguire et al., 1993; Rijli et al., 1993). In addition, malformations in other tissues including kidneys, radius and sternum were observed (Schorle et al., 1996; Zhang et al., 1996b). The overlapping patterns of expression between *Hox* and *Ap2* and their related functions especially in NCC suggested genetic interactions between these genes and is consistent with the discovery of an AP2-regulated enhancer element in *Hoxa2* (Maconochie et al., 1999). This enhancer is responsible for the correct expression of *Hoxa2* in the NCC of branchial arch 2 but not in the hindbrain suggesting a role for AP2 as a tissue-specific regulator. AP2 cooperates with two as yet unidentified factors that bind to two *cis*-acting elements

in the *Hoxa2* NCC enhancer, thus the exact role of the AP2 family members in this regulatory machinery is not clear.

2.3.7 *GATA1*

GATA1, GATA2 and GATA3 are zinc-finger-containing transcription factors that recognize a DNA-core consensus of 5' GATA 3'. GATA1 expression is restricted to hematopoietic lineages and to the sertoli cells of the testis (Yamamoto et al., 1997). *HoxB* gene expression patterns in the hematopoietic system led investigators to study the role of GATA1 in the regulation of *Hox* expression in this tissue. Deletional and mutational analyses coupled with transactivation assays in erythroid cell lines and EMSA analysis using nuclear extracts from erythroleukemic cell lines revealed a GATA1-regulated enhancer in the 5' regulatory region of the human *HOXB2* gene (Vieille-Grosjean and Huber, 1995).

2.3.8 *Polycomb (PcG) and Trithorax (TrxG) group proteins*

PcG and TrxG were first identified in *Drosophila* as multiprotein complexes implicated in transmitting cellular memory. The initial activation or repression of the homeotic genes are mediated by transiently expressed upstream regulators (as described above), however the maintenance of the inherited repressed or activated expression state through cell division is mediated by PcG and TrxG, respectively. In accordance, PcG and TrxG null mutants show normal initial *Hox* expression early in embryonic development, however at later developmental stages, misexpression of *Hox* genes becomes evident. Thus, among the phenotypes of *PcG* and *TrxG* mutants are several homeotic

transformations (Akasaka et al., 1996; Core et al., 1997; Takihara et al., 1997; van der Lugt et al., 1996). PcG and TrxG have been shown to contain multiple proteins as suggested genetically by synergism between their different mutants, coimmunolocalization of these proteins on polytene chromosomes in *Drosophila* and nuclear colocalization in mammals. PcG and TrxG protein components are expressed in a tissue-specific manner and differentially through out development. Hence, it's believed that distinct PcG and TrxG complexes specifically regulate distinct target genes (Satijn and Otte, 1999, and references therein).

The mechanism by which these protein groups mediate their functions is not completely understood, however it is believed to involve rearrangement of the higher order chromatin structure. Several models exist to explain the mode of action of the PcG genes. These include compacting of the chromatin (Paro, 1990), interference between enhancer and promoter (Pirrotta and Rastelli, 1994), formation of an inactive promoter-silencer complex (Bienz and Muller, 1995), sequestering target genes into nuclear compartments (Paro, 1993) or, most likely, repositioning of nucleosomes (Pirrotta, 1997a; Pirrotta, 1997b). PcG proteins recognize a *cis*-acting element in the regulatory regions of downstream targets known as a PRE or polycomb response element. The PRE spans several hundred bp and contains multiple elements. TrxG also bind to PREs, however the binding sites of PcG and TrxG within the PRE are distinct (Tillib et al., 1999). Only recently, the DNA-binding component of the PcG complex has been identified as the *pleiohomeotic* (*Pho*) gene product in *Drosophila* (Brown et al., 1998), the homologue of the mammalian ubiquitously expressed zinc-finger transcription factor Yin-Yang 1 (YY1) (Riggs et al., 1993). Mutation of the PHO binding sites in the

engrailed PRE were shown to abolish its repressive function, suggesting that binding of PHO is required for PcG activity. However, multimerization of PHO binding sites could not repress the expression of a reporter gene in the same manner as the PRE (Brown et al., 1998). This result implied the existence of other DNA-binding proteins within the PcG complex that function along with PHO or YY1. Data supporting this hypothesis came from a study analyzing repression of *Hox* genes by the Gap gene product Hunchback (HB). Genetic analyses implied dMi 2, the *Drosophila* homologue of the dermatomyositis-specific human auto-antigen Mi 2, in the HB- and PcG-mediated repression (Kehle et al., 1998). Interestingly, mammalian Mi 2 is a component of NURD, a complex that contains nucleosome remodeling and histone deacetylase activities. This is consistent with the idea that PcG proteins repress transcription by modifying chromatin, deacetylating its histones and remodeling its structure to inhibit access of transcription factors (Kehle et al., 1998; Wade et al., 1998; Xue et al., 1998; Zhang et al., 1998a). Other components of the TrxG and PcG complexes have been shown to possess chromatin modifying properties. For instance, BRAHMA, a TrxG component is the mammalian homologue of the yeast SWI2/SNF2 chromatin remodeling protein (Tamkun et al., 1992); similarly, GAGA, the product of the *Trx-like* gene, functions with NURF (nucleosome remodeling factor) to displace nucleosomes (Tsukiyama and Wu, 1995).

The components of the PcG and TrxG complexes have been conserved through evoluuion and found to exist in *C. elegans*, *Xenopus*, chicken, mouse and man (Satijn and Otte, 1999). In the mouse, null mutation of PcG genes *Bmi1*, *mel18*, *M33*, *Rae28* and *eed* causes posterior transformations in the axial skeleton that correlates with anteriorized expression of some *Hox* genes (Akasaka et al., 1996; Core et al., 1997; Takihara et al.,

1997; van der Lugt et al., 1996). Similarly, null mutation of the TrxG gene *Mll* results in posteriorized *Hoxa7* and *Hoxa9* in heterozygotes and no expression in homozygotes (Yu et al., 1995). These data suggest a regulatory role of PcG and TrxG in the regulation of mammalian *Hox* gene expression; however to date no direct evidence, such as the characterization of a PRE in a mammalian *Hox* gene regulatory region, has been provided to support this hypothesis.

3. THE HOX PROTEIN AS A TRANSCRIPTION FACTOR

It is widely believed that HOX proteins achieve their developmental program by acting at the level of transcription, regulating the expression of downstream effector genes. Most HOX protein functions are mediated through specific binding to DNA, *via* the homeodomain; however, some DNA-binding-independent functions have been reported for some homeoproteins. For example, the *Drosophila* segmentation gene *Fushi tarazu* encodes a homeodomain-containing transcription activator, FTZ, that can alter gene expression without binding to DNA (Copeland et al., 1996; Fitzpatrick et al., 1992). Similar to the wild-type protein, the mutant protein lacking the homeodomain (FTZ ΔHD) leads to loss of odd-numbered para-segments when ectopically expressed throughout the blastoderm embryo, resulting in an "anti-*ftz*" phenotype. The DNA binding-independent functions of FTZ ΔHD could be mediated through interactions with other proteins. Similarly, transcriptional repression by the homeoproteins MSX1 and PBX1 does not require their homeodomains (Catron et al., 1995; Lu and Kamps, 1996a). Among HOX proteins that may function in a DNA binding-independent manner are the products of splice variants that do not encode the homeodomain as in the case of *Drosophila*

Antennapedia (*Antp*) (Bermingham and Scott, 1988), *Ultrabithorax* (*Ubx*) (Kornfeld et al., 1989; O'Connor et al., 1988) and mouse *Hoxa1* (LaRosa and Gudas, 1988). However, no data supporting this hypothesis have yet been provided.

In order to decipher the molecular mechanisms underlying the functions of the *Hox* genes during development, it is essential to characterize downstream targets which are regulated by the HOX proteins and understand how HOX proteins activate or repress transcription. To date, very little is known about HOX target genes, except for *Hox* genes themselves that are autoregulated by their own products or crossregulated by the products of other *Hox* genes (as described above). Few other HOX targets have been suggested and have contributed to our knowledge on HOX functions, and these include genes encoding cell adhesion molecules (Edelman and Jones, 1995; Gould and White, 1992; Graba et al., 1992; Jones et al., 1992), growth factors (Immerglück et al., 1990; McWhirter et al., 1997; Reuter et al., 1990), transcription factors (Guazzi et al., 1994; Raman et al., 2000b; Vachon et al., 1992; Wagner-Bernholz et al., 1991), tumor suppressor genes (Raman et al., 2000a; Tomotsune et al., 1993) and cell-cycle regulators (Bromleigh and Freedman, 2000). We will only understand how HOX proteins exert their various effects when additional stage- and tissue-specific target genes are identified.

3.1 HOX protein functional domains

3.1.1 The Homeodomain

The homeodomain is the DNA-binding structure of the HOX proteins (Scott et al., 1989). It is encoded by the 180 bp sequence of the homeobox and is thus composed of 60 aa residues. The amino acid sequence of the homeodomain has been conserved to a high

degree by evolutionary pressure; the human HOXA7 homeodomain, for instance, differs in only 1 out of 60 amino acids from that of *Drosophila* Antennapedia (ANTP), despite the fact that the two species separated more than 500 million years ago. Similar to sequence similarities, some unique features of the homeodomain structure, such as the backbone of the helix-turn-helix motif, have been also conserved and are superimposable with those found in yeast transcriptional regulators and the more distant prokaryotic DNA-binding proteins (for review, see Gehring et al., 1994b). Nuclear magnetic resonance (NMR) studies analyzing the solution structure of the ANTP homeodomain first revealed that the homeodomain is composed of three α-helices tightly folded in a globular structure (Billeter et al., 1990; Qian et al., 1992). Helix 1 (residues 10-21) is separated from helix 2 (residues 28-38) by a loop, and a helix-turn-helix motif links helix 2 to helix 3 (residues 42-59). Another important feature of the homeodomain is a flexible N-terminal arm (residues 1-8) that precedes helix 1. NMR studies of the ANTP homeodomain bound to DNA (Billeter et al., 1993; Otting et al., 1990), together with X-ray crystallography of the engrailed (EN) (Kissinger et al., 1990) and Mat α2 (Wolberger et al., 1991) homeodomain-DNA complexes demonstrated that the three-dimensional structure of all three homeodomains is very similar. This was unexpected when comparing the Mat α2 and ANTP homeodomains since they share only 28% sequence identity and differ from each other by the presence of a three aa insertion in the loop of the Mat α2 homeodomain. Such an insertion is characteristic of the TALE (three aa loop extension) class of homeoproteins that also includes the HOX cofactors PBX and MEIS proteins (see below).

Mobility shift assays, transactivation assays and footprinting have determined the core DNA motif recognized by most homeoproteins examined as the sequence 5' TAAT 3' (for review, see Gehring et al., 1994a). The structural studies mentioned above used such a motif and determined the points of contacts between the homeodomain and DNA. In summary, four major sites in the homeodomain are responsible for DNA recognition, and these include helix 3, the N-terminal arm and residues in the loop and at the beginning of helix 2. Helices 1 and 2 are aligned in an anti-parallel arrangement above the DNA and are nearly perpendicular to the major groove DNA-backbone. Helix 3 lies in the major groove and is roughly parallel to the groove. The N-terminal arm reaches behind the phosphate backbone to contact bases via the minor groove (Fig. 4A).

Helix 3 is the recognition helix that mediates most of the specific intermolecular contacts. Residue 50 in helix 3 plays a dominant role in determining the sequence recognized by the homeodomain. It establishes a hydrogen bond with the variable base 3' to the TAAT core and as such contributes to DNA-binding specificity. In ANTP, as for all HOX proteins, this position is occupied by glutamine. Other homeoproteins have different residues at this position. For example, Bicoid (BCD) have a lysine instead of glutamine and the substitution of lysine to glutamine switches the BCD DNA binding specificity to that of ANTP, both in heterologous expression systems and in *in vitro* binding assays (Hanes and Brent, 1989; Treisman et al., 1989). In addition to glutamine 50, the invariant asparagine 51, isoleucine 47 and methionine 54 are also important in DNA-protein interactions. Asparagine 51 makes an important contact at position 3 of the TAAT core and through water molecules contacts position 2 as well. Mutation of this single residue has been shown to be detrimental for the monomer DNA-binding of

different homeoproteins including HOX, PBX and MEIS (Billeter et al., 1996; Billeter et al., 1993; Kissinger et al., 1990; Lu et al., 1994; Passner et al., 1999; Piper et al., 1999; Shanmugam et al., 1999; Wolberger et al., 1991). Both isoleucine 47 and methionine 54 contact DNA through hydrophobic bonds mediated by their side chains, with Ile 47 contacting T3 and A4, and Met 54 the first residue 3' to the TAAT core.

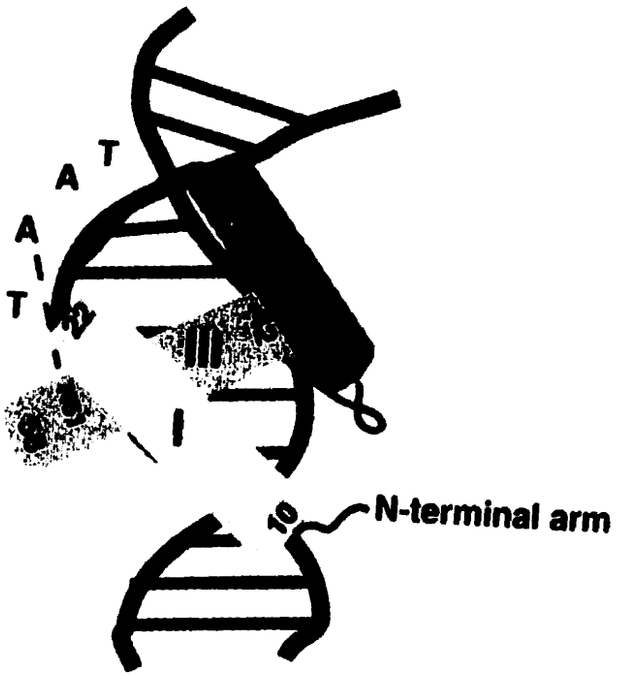
The flexible N-terminal arm is disordered in solution; however, upon binding to DNA it attains an ordered conformation and establishes base-specific contacts with the minor groove. Genetic studies in *Drosophila* have demonstrated that the N-terminal arm contributes significantly to the functional specificity of the homeoproteins. Ectopic expression of ANTP-SCR (Sex Comb Reduced) chimeras in transgenic flies indicated that the functional specificity of the chimera is determined by the composition of the first six residues of its homeodomain (Furukubo-Tokunaga et al., 1993). Similar results were obtained for Ultrabithorax (UBX) and Deformed (DFD) HOX proteins (Chan and Mann, 1993; Lin and McGinnis, 1992; Mann and Hogness, 1990). The two residues in the N-terminal arm that contribute most to the minor groove contacts are arginines 3 and 5, as shown from the structural studies on the homeodomains of EN and ANTP (Billeter et al., 1993; Kissinger et al., 1990; Otting et al., 1990). In the EN homeodomain, Arg 3 contacts the second position of the TAAT core while Arg 5 contacts the first position. Biochemical studies indicate that these two residues are crucial for the specificity of DNA-binding. ANTP with Arginines at positions 3 and 5 binds a TAAT core motif (Beachy et al., 1988; Desplan et al., 1988; Ekker et al., 1992; Ekker et al., 1991; Florence et al., 1991; Hoey and Levine, 1988; Phelan and Featherstone, 1997) while, more 5' in the *Hox* cluster, ABD-B HOX proteins with lysine at position 3 prefer binding to TTAT

Figure 4: Structures of HOX and HOX•PBX homeodomains on DNA.

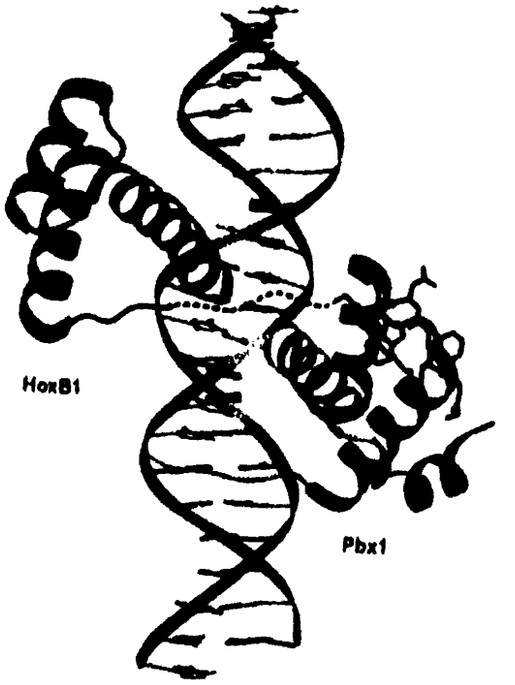
(A) Schematic representation of monomeric HOX homeodomain bound to DNA. The homeodomain is folded into three α-helices and a flexible N-terminal arm. Helices I and II are aligned in an anti-parallel arrangement above the DNA, nearly perpendicular to the major groove, and helix III lies in the major groove and is parallel to the groove. The N-terminal arm contacts the minor groove. Helix I extends from residues 10 to 22 of the homeodomain, helix II from residues 28 to 37, helix III from residues 42 to 58 and the N-terminal arm precedes helix I and is composed of residues 1 to 9. Asparagine 51 (N51) in helix III, recognizing the second position of the TAAT core motif, is crucial for DNA-binding.

(B) An illustration taken from (Piper et al., 1999) representing the homeodomains of HOXB1 and PBX in a HOX•PBX heterodimer complex bound to DNA. Both homeodomains bind in a similar manner as in (A) but to opposite faces of the double helix.

A



B



(Benson et al., 1995; Ekker et al., 1994). In addition to conferring DNA-binding specificity, the N-terminal arm has been shown to contribute as well to the affinity of binding of the homeodomain to its consensus sequence. Analysis of the NMR structure of an ANTP homeodomain lacking the N-terminal arm revealed that, while the overall structure of the homeodomain remained unchanged, its DNA-binding affinity was reduced 10 fold relative to the intact homeodomain (Qian et al., 1994). The equilibrium dissociation constant $\{K_D\}$ of the wild-type ANTP homeodomain to its specific binding site was estimated to be around 1.8×10^{-10} M (Affolter et al., 1990). Interestingly, the affinity of binding of HOX proteins to a consensus DNA site, modulated by their N-terminal arm, was shown to correlate with the positions of their genes on the *Hox* cluster (Pellerin et al., 1994). Thus, one can speculate that subtle differences along the cluster in both DNA-binding specificity and affinity may constitute features of the "*Hox* code" that contribute to the selective functions of the mammalian HOX proteins during development.

The ability of the N-terminal arm to modulate binding to DNA has been recently shown to be regulated by post-translational modifications, such as phosphorylation. Two reports presented reciprocal findings with regard to the phosphorylation of the N-terminal arm and consequent increased or decreased DNA-binding. In the case of the FTZ homeodomain, phosphorylation of threonine at position 7 of the N-terminal arm by PKA was shown to be required for normal protein activity. Substitution of threonine 7 by the unphosphorylatable alanine inhibited the activity of the protein in transgenic analysis (Dong et al., 1998). Conversely, PKA phosphorylation of the N-terminal arm of SCR led

to inhibition of both DNA-binding, *in vitro*, and activity of the protein in transgenic flies (Berry and Gehring, 2000). In contrast, dephosphorylation by the protein phosphatase 2A (PP2A), in response to RAS signaling, positively modulated SCR DNA-binding ability and function. These studies provide one example of how cell signaling could modify HOX function.

Besides major groove and minor groove contacts mediated, respectively, by the recognition helix and the N-terminal arm, residues in the loop between helices 1 and 2 and at the beginning of helix 2 are also important for homeodomain-DNA interactions. In the case of the ANTP homeodomain, these include tyrosine 25, arginine 28 and arginine 31 (Billeter et al., 1993; Otting et al., 1990).

The homeodomain exerts multiple functions. In addition to mediating binding to DNA, it is responsible for the nuclear localization of some homeoproteins. Careful characterization of the nuclear localization signals was provided in only a few studies. A summary of the homeodomains bearing one or more NLS is listed in Table 1.

Homeodomains bearing one or more NLS

Protein	#NLS in HD	NTA	Helix 3	Cooperative	References
LHX3	3	yes	yes	yes	(1)
NKX2.2	2	yes	yes	yes	(2)
NKX2.5	1	yes	na	na	(3)
PDX1	1	no	yes	na	(4, 5)
TTF1	2	yes	yes	yes	(6)
TTF1	2*	yes	?	yes*	(7)
IPF1/IDX1	1+	?	?	na	(8, 9)
TST1/OCT6	1	yes	na	na	(10)
PAX6	1	yes	na	?	(11)
EXD	1	yes	na	na	(12)
PBX1	1+	?	?	na	(13)
PBX1	2	yes	yes	yes	(14)
DLX3	2	yes	na	yes	(15)
TGIF2	1	?	?	na	(16)
KN1	1	?	?	?	(17)
HOXA5	1+	no	?	?	(18)
ANTP	1	no	yes*	na	(19)

In these studies, the NLS were finely mapped and, where noted, shown to be contained in the N-terminal arm "NTA" and/or helix 3. In some instances, the NLS were demonstrated to function in a "cooperative" manner. "na", not applicable; "?", not investigated; *, implied; +, 1 or more NLS

(1) (Parker et al., 2000); (2) (Hessabi et al., 2000); (3) (Kasahara and Izumo, 1999); (4) (Moede et al., 1999); (5) (Hessabi et al., 1999); (6) (Christophe-Hobertus et al., 1999); (7) (Ghaffari et al., 1997); (8) (Stoffers et al., 1998); (9) (Lu et al., 1996); (10) (Sock et al., 1996); (11) (Carriere et al., 1995); (12) (Abu-Shaar et al., 1999); (13) (Berthelsen et al., 1999); (14) (Saleh et al., 2000a); (15) (Bryan and Morasso, 2000); (16) (Imoto et al., 2000); (17) (Meisel and Lam, 1996); (18) (Zhao et al., 1996); (19) (Gibson et al., 1990).

The homeodomain has been implicated as well in transcriptional regulation. For example, repression by HOXA7, HOXD8 and MSX1 proteins is mediated by their respective homeodomains and the modulatory action of their N-terminal arms (Schnabel, 1996; Zappavigna et al., 1994; Zhang et al., 1996a). Similarly, transcriptional activation by HOXD9 is mediated through protein-protein interactions involving its homeodomain and the high mobility group protein 1 (HMG1) (Zappavigna et al., 1996). Such an interaction is believed to enhance the DNA-binding ability of the protein and facilitates its access to downstream targets, mediated by the architectural role of HMG proteins in chromatin structure.

Not all activation and repression functions of HOX proteins are contained within their homeodomains. Activation and repression domains have been characterized as separate functional entities in both the N- and C-terminal portions of HOX proteins, endowing them with their transcriptional regulatory properties (see below).

3.1.2 *HOX activation and repression domains*

Little is known about the functions of the N- and C-terminal regions of the HOX proteins. Conservation of these regions among the vertebrate *Hox* genes and between these genes and their *Drosophila* orthologs is minimal and essentially restricted to the YPWM (also known as the pentapeptide or hexapeptide) motif (see below), and a few N-terminal aa (in some cases known as the N-terminal motif or the octapeptide) (Boncinelli et al., 1985; Mavilio et al., 1986; McGinnis and Krumlauf, 1992). However, the functions of these regions have been shown to be absolutely essential in very stringent functional tests. For instance, the N-terminus of the mouse HOXA5 protein was demonstrated to be

required to induce SCR-like homeotic transformations in the fly and to activate the expression of the *forkhead* (*fkh*) gene, an SCR-downstream target (Zhao et al., 1993; Zhao et al., 1996). These *in vivo* functions were later attributed to the presence of a transcriptional activation domain in the HOXA5 N-terminus that was finely mapped to the first 39 aa of the protein, a serine-rich region that includes the octapeptide. Similarly, the mouse HOXD4 protein was shown to activate transcription via an N-terminally located activation domain. This domain is proline-rich and does not include the conserved N-terminal motif. Fusion of the HOXD4 activation domain to the GAL4 DNA-binding domain leads to activation of transcription from GAL4-responsive reporters (Rambaldi et al., 1994; Saleh et al., 2000b). Another proline-rich activation domain was characterized in the human HOXB1 protein. As for HOXA5 and HOXD4, the HOXB1 activation domain is within the N-terminal region of the protein (Di Rocco et al., 1997). Despite being poorly conserved in the HOXB1 *Drosophila* ortholog LAB, this region was shown to be absolutely required for the rescue of the *lab* null phenotype by HOXB1 (Lutz et al., 1996), suggesting that the function, but not the sequence, of the activation domain has been conserved across species. The N-terminal region of other HOX proteins were also shown to harbor activation domains and these include HOXA7, HOXB7, HOXD8 and HOXD9 (Chariot A, 1999; Schnabel, 1996; Viganò et al., 1998; Zappavigna et al., 1994). In addition, the activation domain of HOXB3 was characterized, however in this case, it is located in the C-terminus of the protein (Viganò et al., 1998).

Most of these activation domains do not share similarities in their sequence or even in their amino acid content or character. Nevertheless, recent evidence suggest that

HOX proteins may regulate transcription in similar fashion. In two cases, HOXB7 and HOXD4, transcriptional activation is achieved through the recruitment of coactivators with HAT activity by the HOX activation domains (Chariot A, 1999; Saleh et al., 2000b). This mechanism of regulation has also been demonstrated for other homeoproteins including PIT-1 and PDX1 (Asahara et al., 1999; Xu et al., 1998). Interestingly, for all the homeoproteins analyzed to date, the coactivator in play is the CREB-binding protein (CBP), suggesting that homeoproteins may function in a conserved manner to activate transcription.

In certain cellular environments and promoter contexts, some HOX proteins bearing activation domains were shown to function as potent transcriptional repressors. HOXA7 and HOXD8 are examples. As mentioned above, the activation domains of these two proteins are in their N-termini; repression domains have been characterized in both cases within their homeodomains and in an acidic region at the C-terminus of HOXA7 (Schnabel, 1996; Zappavigna et al., 1994). Repression domains were also mapped in HOXB4 and HOXA9 C-termini and the potency of their repression function was shown to be modulated by residues in the N-terminal arm of their homeodomains (Schnabel. 1996).

When do these proteins function as activators or repressors? One possible scenario would be that in response to cell signaling cues, these proteins would favor interactions with coactivators over corepressors, or *vice versa*. This could result from post-translational modifications of the proteins themselves or of the coregulators, altering their affinity towards each other. Alternatively, interactions with other stage- or tissue-specific factors would lead to a conformational change in the HOX proteins resulting in

the exposure of one effector domain versus the other and its access to coregulators and the general transcription machinery. The situation becomes more complex in the presence of HOX DNA-binding partners such as PBX, with transcriptional effector domains of its own.

3.2 HOX protein DNA-binding partners

Three aspects of DNA-binding by the HOX proteins suggested the requirement of interaction with a DNA-binding partner for site-specific recognition of downstream targets. First, the homeodomain is highly conserved and recognizes similar binding sites with only modest preference, suggesting that HOX proteins alone could not discriminate well between targets (Corsetti et al., 1992; Dessain et al., 1992; Ekker et al., 1992; Pellerin et al., 1994). Second, the HOX core consensus sequence is small and is widely distributed in the genome, suggesting that not all of these sites are used. Third, although the K_D of HOX binding to a consensus site is in the nanomolar range, the affinity of binding to non-specific sites is relatively strong (Affolter et al., 1990; Ekker et al., 1991), implying the requirement for a cofactor to direct binding to only specific sites. Supporting the presence of a cofactor, genetic studies in the fly indicated that non-DNA-binding regions flanking the homeodomain are also required to achieve functional specificity, presumably through protein-protein interactions (Gibson et al., 1990; Kuziora and McGinnis, 1991; Lin and McGinnis, 1992; Mann and Hogness, 1990). A major breakthrough came in 1990 when a *Drosophila* mutant, *extradenticle* (*exd*), was shown to have altered segmental identity with no change in *Hox* gene expression (Peifer and Wieschaus, 1990). Maternal overexpression of *exd* was demonstrated to rescue the *exd*

phenotype indicating that in the absence of EXD, HOX proteins alone are not sufficient to specify segment identity. This suggested that EXD is a potential HOX cofactor required to cooperate with HOX for specific regulation of target genes. Cloning of *exd* demonstrated that its product is a homeoprotein homologous to the human proto-oncogene *PBX1* (Kamps et al., 1990; Nourse et al., 1990; Rauskolb et al., 1993). EXD was subsequently shown to cooperate with *Drosophila* HOX proteins to regulate target genes (Chan et al., 1994; Rauskolb and Wieschaus, 1994; van Dijk and Murre, 1994) and in parallel PBX was demonstrated to function as a cofactor for mammalian HOX proteins from paralogous groups 1 to 10 (Chang et al., 1996; Shen et al., 1997a). Today, there are two cofactor families that regulate the functions of mammalian HOX proteins: The PBX family and the MEIS/PREP1 family. Both cofactor groups belong to the TALE class of homeoproteins, with three aa loop extension in their homeodomains (Burglin, 1997) (Fig. 5). This characteristic TALE proved to be essential for the function of these homeoproteins as HOX cofactors as was revealed from structural studies analyzing HOX•PBX homeodomain complexes on DNA (Passner et al., 1999; Piper et al., 1999) (see below).

3.2.1 *The PBX family*

The proto-oncogene *PBX1*, pre-B cell acute lymphoblastic leukemia (ALL)-related factor, was identified at the chromosomal breakpoint of t(1;19) translocalion found in 25 % of all childhood pre-B cell ALL (Kamps et al., 1990; Nourse et al., 1990). This translocation results in the expression of two novel chimeric mRNAs with the same 5' sequence, derived from the *E2A* gene, fused to two differentially-spliced mRNAs

variants from the *PBX1* gene (*PBX1a* or *PBX1b*). The *E2A* gene encodes the Eκ box enhancer-binding transcriptional activator, that belongs to the basic helix-loop-helix (bHLH) family of transcription factors. The E2A-PBX fusion proteins encompass the activation domains of the E2A protein within its N-terminal two thirds fused to most of PBX1A or PBX1B at position 89. The oncoprotein E2A-PBX is therefore believed to contribute to ALL by hyper-activating PBX-responsive genes. The distribution pattern of PBX1 is in accordance with this hypothesis. The *PBX1* gene is expressed in most fetal and adult tissues and all cell-lines examined; however, it is not expressed in lymphoid cell-lines (Monica et al., 1991; Roberts et al., 1995). The presence of E2A-PBX in pre-B cells may thus lead to transformation by altering the expression of PBX1 target genes that should be otherwise inactive or less active in these cells. Site selection studies determined the core DNA-binding site of the PBX homeodomain to be 5' TGATTGAT 3' and showed that while E2A-PBX strongly activates transcription *in vivo* from this site, PBX does not (Lu et al., 1994). In chapters 2 and 3, we offer additional mechanisms by which E2A-PBX may contribute to ALL. We and others have shown that, in the absence of MEIS/PREP1, region 1-89 of PBX1 functions in the inhibition of its nuclear localization; thus E2A-PBX, lacking residues 1-89 of PBX, would be constitutively nuclear in all tissues (Berthelsen et al., 1999; Saleh et al., 2000a). A previous study examining the subcellular localization of E2A-PBX supports this model (LeBrun et al., 1997). In addition, we showed (chapter 3) that region 1-89 contains a potent TSA-insensitive repression domain (Lu and Kamps, 1996a; Saleh et al., 2000b). Thus, the oncogenicity of E2A-PBX may be due to both the loss of a repression domain, as well as to the recruitment of HATs by the E2A activation domains (Massari et al., 1999).

Two other *PBX* genes were identified on the basis of their high homology to *PBX1* and were named *PBX2* and *PBX3*. *PBX1*, *PBX2* and *PBX3* genes are not clustered and map to human chromosomes 1, 3 and 9, respectively (Monica et al., 1991). Similar to *PBX1*, *PBX3* produces two alternatively-spliced RNA variants that translate into two proteins with different C-termini, a feature not observed for *PBX2*. PBX2 and PBX3 proteins share 92% and 94% identity with PBX1, respectively, over a 266 aa region within and flanking their homeodomains (Monica et al., 1991). Regions of extensive divergence are present in their N- and C-termini. PBX2 and PBX3 are ubiquitously expressed but, unlike PBX1, are also present in lymphoid lineages. Post-transcriptional modifications of PBX proteins were shown to differentially modulate their accumulation levels in EC cells (P19) in response to RA. In addition, the different splice-variants showed different levels of accumulation (Knoepfler and Kamps, 1997). This suggests that external stimuli as well as differential splicing in *Pbx* transcripts may discriminate between PBX proteins and constitute two important aspects that regulate their tissue-specific functions. The high conservation among the *Pbx* genes together with their wide range of expression that parallels that of many *Hox* genes suggest a general but overlapping function of the PBX proteins and is consistent with them acting as HOX cofactors (Monica et al., 1991; Roberts et al., 1995).

PBX proteins and their homologs from *Drosophila* (EXD), from *C. elegans* (CEH-20) and the recently identified "Lazarus or PBX4" from Zebrafish (Popperl et al., 2000; Vlachakis et al., 2000) compose the PBC class of homeoproteins (Burglin and Ruvkun, 1992). In addition to conservation in their TALE homeodomains, they share two highly conserved regions N-terminal to the homeodomain referred to as the PBC-A and

PBC-B domains (Burglin and Ruvkun, 1992). Functional dissection of the PBX1 protein revealed three repression domains in its N-terminus (Lu and Kamps, 1996a) that span not only parts of PBC-A and PBC-B but also a polyalanine stretch in between, conserved in mammals and flies. Polyalanine stretches have been associated with repression domains in other homeoproteins (Han and Manley, 1993a; Han and Manley, 1993b; Licht et al., 1990; Licht et al., 1994; Licht et al., 1993). Further analysis of the role of PBC-A and PBC-B in other species needs to be performed before associating with them the repression function. Three other functions have been attributed to the PBC-B domain. First, a 25 residue predicted α-helix within the PBC-B region has been shown to inhibit binding by the PBX homeodomain through intramolecular interactions (Calvo et al., 1999; Lu and Kamps, 1996b; Neuteboom and Murre, 1997; Peltenburg and Murre, 1997; Saleh et al., 2000a). Second, a dimerization interface N-terminal to the inhibitory helix, was characterized in PBC-B that allows PBX family members to homodimerize and heterodimerize (Calvo et al., 1999). Third, a nuclear export function has been attributed to this region in the EXD protein but not in PBX (Abu-Shaar et al., 1999; Berthelsen et al., 1999). PBC-A, on the other hand, has been shown to mediate interactions with the homeoproteins MEIS and PREP1 (Berthelsen et al., 1998b; Chang et al., 1997b; Knoepfler et al., 1997) (see below).

The high homology between PBX and EXD (71%) and the role of EXD in *Drosophila* as a cofactor that cooperates with HOX functions (Mann and Chan, 1996) suggested not only that PBX proteins can act as HOX cofactors but also that HOX proteins expressed in lymphoid lineages are required for the oncogenicity by E2A-PBX. A recent report demonstrated a collaboration between the E2A-PBX oncoprotein and

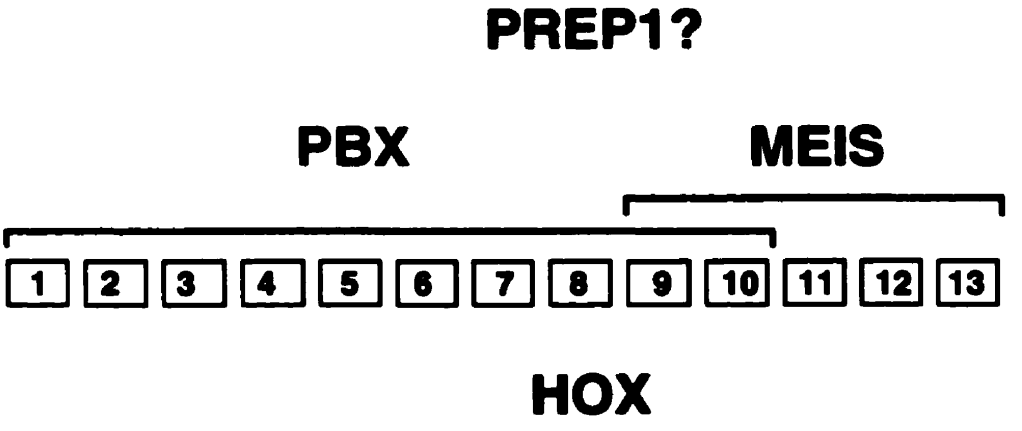
HOXA9 in cellular transformation. Overexpression of E2A-PBX together with HOXA9 in primary bone marrow cells, followed by the transplantation of these cells in syngenic mice led to the induction of an aggressive form of acute leukemia (Thorsteinsdottir et al., 1999). A flurry of studies provided evidence supporting cooperative binding between PBX and HOX proteins from paralogous groups 1 to 10 but not from the remaining groups 11, 12 and 13 (Chang et al., 1996; Shen et al., 1997a). The domains involved in such an interaction were mapped to the conserved YPWM motif in HOX from groups 1 to 8 or the conserved tryptophan for groups 9 and 10 (Chang et al., 1995; Lu et al., 1995; Phelan et al., 1995; Shen et al., 1997a), and to the homeodomain in PBX (Chang et al., 1997a; Green et al., 1998; Lu and Kamps, 1996b). In addition, the role of PBX in modifying HOX DNA-binding specificity and affinity was also extensively investigated (see below). The demonstration that PBX is required to direct segmental expression of the *Hoxb1* gene to r4, *via* an autoregulatory loop dependent upon interaction of HOXB1 and PBX, provided an elegant proof that PBX proteins are *bona fide in vivo* HOX cofactors in mammals (Pöpperl et al., 1995). Gene targeting studies of the *Pbx1* and *lazarus* genes revealed a widespread distribution of patterning defects with no perturbations in *Hox* gene expression, similar to the *exd* mutant (Peifer and Wieschaus, 1990; Popperl et al., 2000; Selleri et al., 2000). Phenotypes were observed only in domains specified by HOX proteins that bear a PBX heterodimerization motif (HOX from paralogous groups 1-10). In the mouse, defects included the absence of a sternum, clavicles and ventral ribs, fusions of hip and shoulder joints, malformations of the second branchial arch-derived structures and abnormal cranial nerve development, as well as malformations in the cervical vertebrae and proximal limbs (Selleri et al., 2000).

Figure 5: HOX DNA-binding partners.

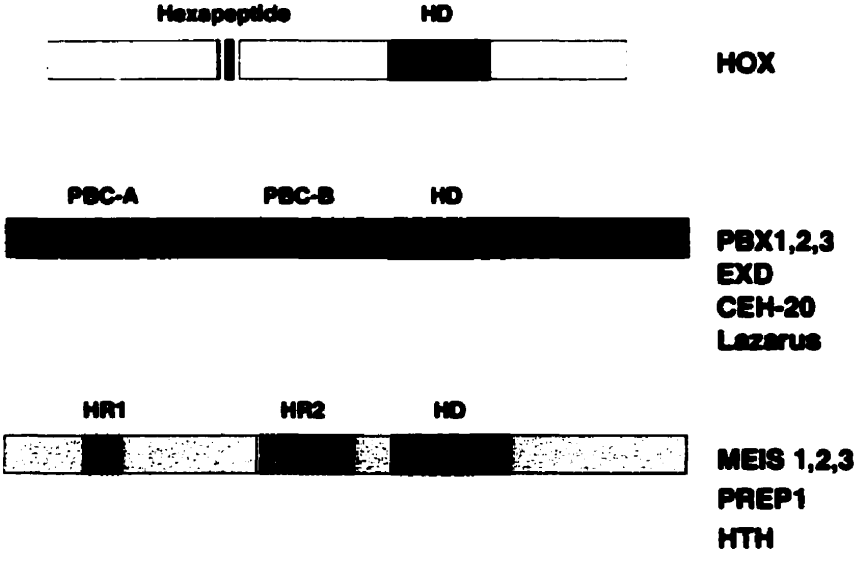
(A) Schematic representation of one general *Hox* cluster, with each box representing a gene, and of the respective interacting cofactor families. PBX interacts with HOX from paralog groups 1 to 10, MEIS interacts with HOX from groups 9 to 13. PREP-1 has not been shown yet to directly bind with any HOX protein on DNA, however it is involved in PBX regulation and in the formation of trimeric complexes with HOX and PBX.

(B) Diagram of a prototypical HOX protein and of its DNA-binding partners from the PBX and MEIS/PREP1 families. The proteins are represented as long rectangles with the homeodomains as black boxes and regions of homology within each family as striped boxes. Homology regions in the N-termini of the proteins are shown. HOX proteins from paralog groups 1 to 8 have the conserved hexapeptide or YPWM motif in their N-termini, separated from the homeodomain by a linker of variable length depending on the group. PBX family members have two conserved regions, PBC-A and PBC-B, and the MEIS/HTH/PREP1 family possesses regions HM1 and HM2 (standing for HTH-MEIS) as homology regions between all the members of the family.

A


PREP1?
PBX
MEIS
1
2
3
4
5
6
7
8
9
10
11
12
13
HOX

B


Hexapeptide
HD
HOX
PBC-A
PBC-B
HD
PBX1,2,3
EXD
CEH-20
Lazarus
HR1
HR2
HD
MEIS 1,2,3
PREP1
HTH

In zebrafish, *lazarus* null embryos presented similar but more severe defects in head segmentation, in the hindbrain, NCC and branchial arches (Popperl et al., 2000). The phenotypes of the *Pbx* null embryos in different species demonstrate its essential role *in vivo* in patterning the body.

3.2.2 *The MEIS/PREP1 family*

The proto-oncogene *Meis1*, *m*yeloid *e*cotropic viral *i*nsertion *s*ite 1, was isolated from myeloid leukemia in BXH-2 mice as the site of viral integration in 15% of tumors (Moskow et al., 1995). It encodes a homeoprotein of the TALE class most closely related within its homeodomain to the PBX/EXD proteins. *Meis1* is alternatively spliced resulting in three MEIS1 proteins, MEIS1A, MEIS1B and MEIS1C (Knoepfler et al., 1997; Moskow et al., 1995). Two other *Meis* genes were subsequently cloned in both mice and Man and were termed *Meis2* (with four protein isoforms) and *Meis3* (Nakamura et al., 1996a; Oulad-Abdelghani et al., 1997; Steelman et al., 1997). *pKnox1* (termed for its similarity to the maize homeobox gene *Knox*), also known today as *Prep1* (for PBX regulatory protein), was later identified as a *Meis*-related gene (Berthelsen et al., 1998b; Chen et al., 1997b; Knoepfler et al., 1997). Besides being highly conserved in their homeodomains, MEIS proteins, PKNOX1/PREP1 and the *Drosophila* MEIS homologue Homothorax (HTH) share two conserved regions N-terminal to their homeodomains referred to as HM1 and HM2 (for HTH-MEIS) (Pai et al., 1998). HM1, HM2 and the conserved N-terminal domains of the maize KNOX are collectively know as the MEINOX domains (Burglin, 1998).

In situ hybridization studies analyzing the expression pattern of *Meis1* and *Meis2* in the developing mouse embryo revealed a wide yet more restricted distribution of MEIS proteins as compared to that of PBX (Cecconi et al., 1997; Huang and Featherstone, 2000; Oulad-Abdelghani et al., 1997). Similar to *Pbx*, *Meis* genes were also shown to be induced by RA in P19 cells (Oulad-Abdelghani et al., 1997). These findings correlate well with the role of RA in regulating HOX functions during embryogenesis: Induction of HOX cofactors is one way to impinge on their activity during body patterning. The first evidence suggesting cooperation between MEIS and HOX proteins came from myeloid leukemias in BXH-2 mice. In 19 out of 20 leukemias with viral insertion in the *Meis1* gene, retroviral activation of either *Hoxa7* or *Hoxa9* was also observed (Nakamura et al., 1996c). This suggested that MEIS and HOXA7/A9 might function as cooperating oncoproteins, possibly through heterodimerization. Various studies provided evidence supporting the role of MEIS in the regulation of HOX function. First, HOX proteins from paralogous groups 9 to 13 were shown to bind DNA cooperatively with MEIS proteins (Shen et al., 1997b) (Fig. 5). Second, simultaneous overexpression of *Hoxa9* and *Meis1* in primary bone marrow cells resulted in myeloid leukemia when these cells were transplanted in syngenic mice (Kroon et al., 1998). Third, nullizygous *Drosophila* embryos with a mutation in the *Meis* fly homologue *Hth* presented defects in antero-posterior patterning without altered expression of the trunk *Hox* genes (Rieckhof et al., 1997). In summary, these data indicate that MEIS is another cofactor for HOX proteins. While PBX cooperates with HOX from groups 1 to 10, MEIS is believed to regulate the functions of ABD-B-like HOX proteins from groups 9 to 13. Interestingly, *Meis* and *Pbx* genes may have evolved from the same ancestral *MEINOX* gene at a point in time when

the two *Hox* clusters, an anterior one and a posterior one, emerged (Burglin, 1998). *Meis* and *Pbx* subsequently evolved to regulate anterior and posterior *Hox* genes, respectively. The interaction of HOX from groups 9 and 10 with both PBX and MEIS proteins raised the question of whether functional HOX•PBX•MEIS trimeric complexes form *in vivo*. HOX proteins from groups 11 to 13 do not possess a YPWM motif (Erselius et al., 1990) nor a conserved tryptophan which mediate interactions with PBX, rather the MEIS-interaction domain in ABD-B-like HOX proteins appear to localize to their N-terminal region (Shen et al., 1997b). Despite the high homology between PKNOX1/PREP1 and the MEIS proteins, PREP1 has not yet been shown to interact with ABD-B-like HOX proteins directly to modulate their binding to DNA.

A separate role of MEIS/PKNOX1/PREP1 proteins is their regulation of PBX functions. MEIS/PKNOX1/PREP1 have been shown to directly bind to PBX. The MEIS-interaction domain in PBX is region from residues 39 to 89 that is deleted in the oncoprotein E2A-PBX. Unlike the HOX•PBX interactions that require binding of the proteins to DNA, the PBX•MEIS/PREP1 complexes are stable both in solution as well as on DNA (Knoepfler et al., 1997) (Berthelsen et al., 1998b; Chang et al., 1997b). The formation of a dimer between PBX and MEIS/PREP1 proteins suggests that on one hand competition for the PBX cofactor may occur between HOX and MEIS/PREP1 proteins and can act as a regulatory mechanism for HOX functions. On the other hand, since the interaction interfaces in PBX with HOX and MEIS/PREP1 are different, the formation of a trimeric complex between all three proteins is feasible. This was demonstrated first for PREP1, interacting with a HOX•PBX heterodimer bound to DNA (Berthelsen et al., 1998a). In addition, the presence of stable PBX•MEIS and PBX•PREP1 heterodimers *in*

vivo indicates the existence of a subset of downstream target genes that are regulated by the PBX•MEIS/PREP1 complexes but not by the HOX•PBX complexes. Some PBX•MEIS/PREP1 targets have been identified and these include the cytochrome p450 17 (CYP 17) gene (Bischof et al., 1998a; Bischof et al., 1998b) and the myogenin gene (Knoepfler et al., 1999) that are regulated by PBX•MEIS, the urokinase plasminogen activator (UPA) gene (Berthelsen et al., 1998b) and the glucagon gene (Herzig et al., 2000) that are under the control of PBX•PREP1 complexes.

Another level of control imposed by MEIS/PREP1 proteins on the PBX function is the regulation of its subcellular localization. The first evidence supporting this regulation came from the *Hth* null embryos where EXD, the *Drosophila* homologue of PBX, was found to be localized exclusively to the cytoplasm (Rieckhof et al., 1997). MEIS proteins were shown to be able to rescue the *Hth* null phenotype and induce cytoplasmic to nuclear translocation of EXD (Pai et al., 1998; Rieckhof et al., 1997). In addition, MEIS directed the nuclear localization of both EXD and PBX in tissue culture (Abu-Shaar et al., 1999; Rieckhof et al., 1997; Saleh et al., 2000a). Similar results were observed for the protein PREP1 (Berthelsen et al., 1999).

3.2.3 *Increased DNA-binding specificity and affinity of the HOX protein following interaction with PBX/MEIS DNA-binding partners*

Interactions with cofactors could modulate the DNA-binding affinity and specificity of the HOX proteins through different mechanisms. First, they can stabilize the protein-DNA complex. Second, they can induce a conformational change in the HOX protein to provoke ordering of a domain or to relieve a negative regulatory structure.

Third, they can mediate recognition of longer DNA sequences that would accommodate binding of a heterodimer or heterotrimer *versus* a HOX monomer. Evidence supporting all mechanisms have been presented. *In vitro* DNA-binding studies indicated longer binding half-lives of HOX•cofactor heterodimers and heterotrimers *versus* that of HOX monomers (Chan et al., 1994; Shanmugam et al., 1999; Shen et al., 1997b). Site-selection studies revealed that heterodimer formation mediated the recognition of longer core sequences: 5' TGATNNAT 3' for PBX•HOX and 5' TTACTGACAG 3' for HOX•MEIS *versus* the 5' TAAT 3' core for the HOX monomer (Chan and Mann, 1996; Chang et al., 1996; Shen et al., 1997b). In the case of HOX•PBX complexes, PBX occupies the 5' half-site TGAT and HOX contacts the more variable 3' half-site NNAT. PBX was demonstrated to modulate the HOX N-terminal arm-DNA contact in the core of the HOX half-site to establish specificity. As such, different HOX were shown to distinguish single nucleotide changes in the HOX half-site both *in vitro* and *in vivo* (Chan and Mann, 1996; Chang et al., 1996; Phelan and Featherstone, 1997). As an example, the autoregulatory element in the fly *lab* gene harbors the sequence TGATGGAT recognized by the LAB•EXD complex. Switching the two central base pairs in the LAB recognition site from GG to TA resulted in switching the expression pattern of a transgene that carries this mutation from LAB-like to that of Deformed (DFD) (Chan et al., 1997). Similarly, converting the SCR•EXD binding site AGATTAATCG in the *forkhead* gene to a consensus site AGATTTATGG recognized by SCR, ANTP and UBX led to the activation of this element by all three proteins *in vivo* (Ryoo and Mann, 1999). In summary, upon interaction with PBX, HOX proteins along the *Hox* cluster showed stepwise preferences for the HOX half-site from TGAT for HOX from groups 1 to 5,

TAAT for HOX from groups 3 to 7 and TTAT for HOX from groups 6 to 10. In contrast, in a HOX•MEIS heterodimer, HOX always contacts a consensus TTAC in the 5' half-site and MEIS binds to the 3' half-site recognizing the core TGACAG (fig) (Chang et al., 1996; Shen et al., 1997b). Interactions between HOX and PBX were shown, by fluorescence spectroscopy, to result in conformational changes in the two proteins modulating their DNA-binding properties and allowing the formation of a cooperative complex (Sanchez et al., 1997). As mentioned above, an inhibitory helix N-terminal to the PBX homeodomain inhibits its DNA-binding ability as a monomer (Calvo et al., 1999; Lu and Kamps, 1996b; Neuteboom and Murre, 1997; Peltenburg and Murre, 1997; Saleh et al., 2000a). Similarly, the conserved YPWM motif within the N-terminus of the HOX protein was suggested to function as an inhibitory domain that blocks the homeodomain and hence the DNA-binding ability of the monomeric HOX (Chan et al., 1996). Mutation of the YPWM motif of the LAB protein resulted in a LAB binding activity that is independent of EXD *in vitro*, and is hyperactive in embryos (Chan et al., 1996). The interaction of HOX and PBX in solution involving the YPWM motif and the PBX homeodomain induces a conformational change that relieves the negative regulatory effect of the inhibitory domains, allowing high affinity DNA-binding. Accordingly, it was proposed that PBX function *in vivo* is to switch HOX proteins from repressors to activators (Pinsonneault et al., 1997). We and others present evidence supporting this hypothesis, whereby the presence of PBX/EXD is required to expose the activation domain of HOX proteins (Li et al., 1999; Saleh et al., 2000b).

3.2.4 *Structure of the PBX•HOX homeodomains on DNA*

The recently solved crystal structures of the HOXB1•PBX homeodomains bound to a consensus DNA sequence TGATTGAT (Piper et al., 1999) and that of UBX•EXD on TGATTTAT (Passner et al., 1999) together with the NMR studies analyzing the structures of HOX•PBX both in solution and on DNA (Jabet et al., 1999; Sprules et al., 2000) came in full agreement with the previously reported data on HOX•PBX interactions and binding to DNA. First, HOX and PBX bind in a head to tail orientation on opposite faces of the DNA double helix, with each homeodomain contacting DNA in a manner similar to what was observed for the ANTP (Billeter et al., 1993) and EN homeodomains (Kissinger et al., 1990) (Fig. 4B). Second, most of the cooperativity arise from interactions between the YPWM motif in the HOX protein and the PBX homeodomain. The YPWM motif is separated from the homeodomain by a linker of variable length: The linker allows the YPWM to form a reverse turn and insert itself in a hydrophobic pocket within the PBX homeodomain. This pocket is composed in part by the three amino acid loop extension (TALE). Third, a region C-terminus to the PBX homeodomain, previously referred to as the PBX cooperativity element (PCE) (Lu and Kamps, 1996b), is unstructured in solution (Jabet et al., 1999; Sprules et al., 2000) but folds into a fourth α-helix upon binding to DNA and packs back against the third helix (Piper et al., 1999). Though it does not contact the YPWM motif, the PCE is required for maximal cooperativity with HOX proteins and for maximal binding by monomer PBX to DNA (Green et al., 1998; Lu and Kamps, 1996b). These results are supported by the crystal structure data from which it is deduced that helix 4 holds helix 3 in an optimal

position for the insertion of the YPWM motif. In addition, helix 4 stabilizes the configuration of helix 3 to result in greater DNA-binding strength (Piper et al., 1999).

These studies greatly improved our understanding of the HOX•PBX recognition of target sequences, however, we still do not know how different linker lengths between the YPWM motif and the homeodomain would affect interactions with PBX and binding to DNA, whether the conserved tryptophan residue in HOX from groups 9 and 10 would interact in the same manner with PBX or how the other HOX DNA-binding partners-MEIS/PREP1-would interact with ABD-B HOX proteins. In addition, we still ignore whether the structure of the HOX•PBX heterodimer on DNA would be different in a trimeric complex where a third cofactor such as MEIS/PREP1 would interact with HOX•PBX in a DNA-binding –dependent or independent manner (as it was recently reported in Ferretti et al., 2000; Jacobs et al., 1999; Shanmugam et al., 1999; Shen et al., 1999; Swift et al., 1998)

4. SUBCELLULAR LOCALIZATION OF PBX1

4.1 General mechanisms of nuclear entry

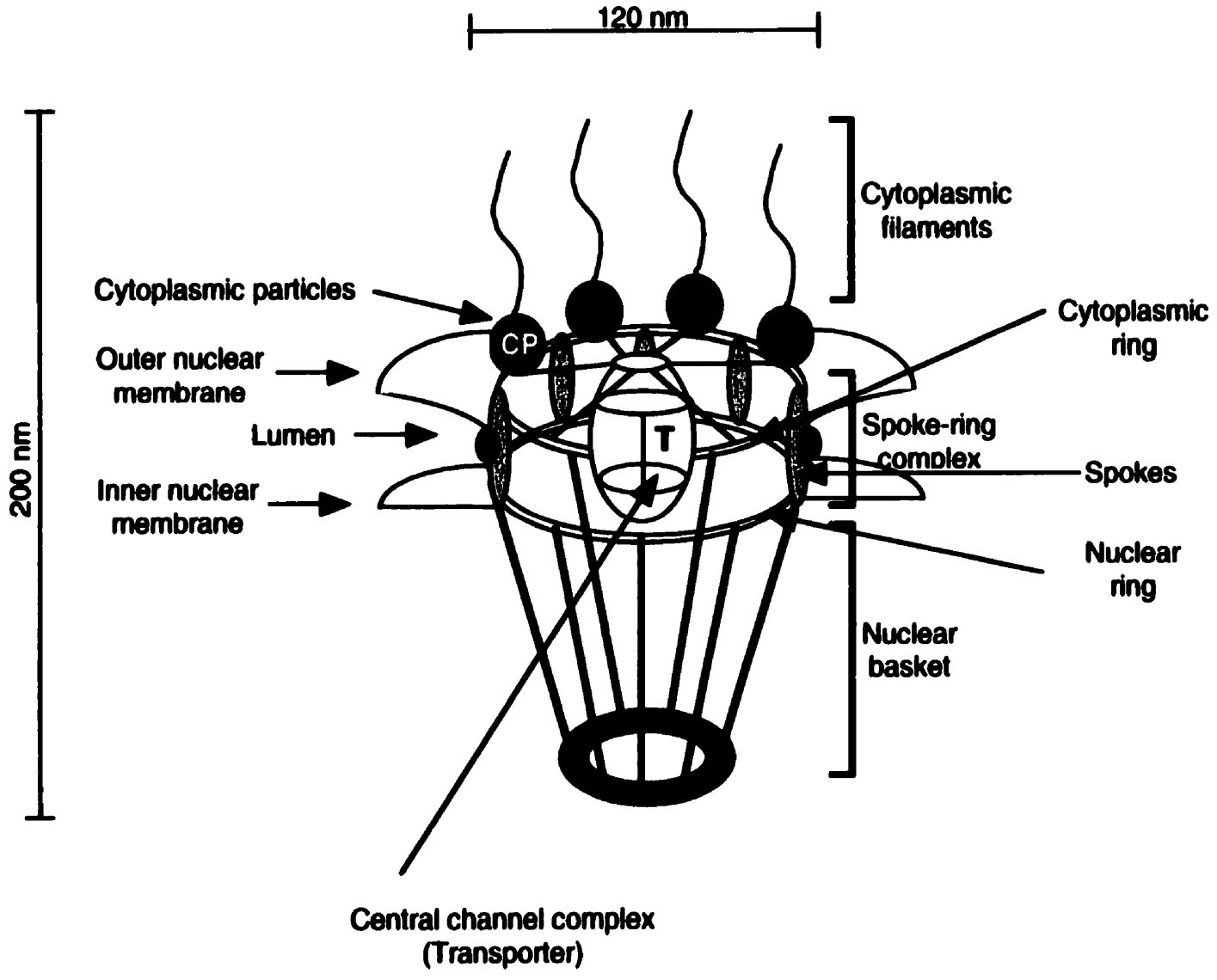
4.1.1 *The nuclear pore complex (NPC)*

In eukaryotic cells, major cellular processes are spatially segregated by the presence of a double membrane system known as the nuclear envelope (NE). This compartmentation gives rise to the need for efficient transport mechanisms of proteins and nucleic acids across the NE. In a growing mammalian cell, more than one million macromolecules per minute are actively transported between cytoplasm and nucleus (Mattaj and Englmeier, 1998). The first electron microscopy (EM) study of the NE

revealed the presence of large proteinaceous structures perforating the envelope, which were later termed "nuclear pore complexes' (NPC) (Callan and Tomlin, 1950). The first evidence suggesting transport through the NPC came from studies examining the ability of microinjected colloidal gold particles to localize to the nucleus (Feldherr, 1965). Subsequent efforts focusing on the detailed three-dimensional architecture, composition and permeability of the NPC led to high resolution models of the vertebrate and yeast NPCs (fig) (Yang et al., 1998) and to the proposal of a mechanism for nucleo-cytoplasmic transport. The NPCs are the sole sites of exchange between the nucleus and the cytoplasm. By EM, they appear like roughly cylindrical structures. Their central part is composed of an octagonal arrangement of eight spokes that is embedded in the NE and that encircles a central channel complex or the transporter (Akey, 1989; Akey, 1990). The transporter has been described in open and closed configurations on both the cytoplasmic and nuclear faces, indicating that it is gated on both sides (Akey, 1990; Akey and Goldfarb, 1989). It mediates bi-directional transport through an aqueous channel of about 9 nm in diameter that allows passive diffusion of ions, metabolites and small proteins (relative molecular mass less than 40-60 KDa) and dilates up to 26-28 nm in diameter to mediate active energy-dependent transport of larger particles (Davis, 1995; Feldherr and Akin, 1990; Pante and Aebi, 1995). Within the lumen of the NE, the spokes are inter-connected by a lumenal ring that attaches the NPC to the NE (Yang et al., 1998). This basic framework is positioned between a cytoplasmic ring and a nuclear ring. The cytoplasmic ring carries eight cytoplasmic filaments that extend up to 50 nm into the cytoplasm.

Figure 6: The nuclear pore complex.

Schematic representation of the three dimensional structure of the vertebrate NPC as described in (Yang et al., 1998).


120 nm
200 nm
Cytoplasmic filaments
Cytoplasmic particles
CP
Outer nuclear membrane
Lumen
Inner nuclear membrane
T
Cytoplasmic ring
Spoke-ring complex
Spokes
Nuclear ring
Nuclear basket
Central channel complex
(Transporter)

The nuclear ring carries the nuclear basket, which is composed of eight thinner fibrils of around 100 nm in length that join in a terminal ring (Goldberg and Allen, 1992; Jarnik and Aebi, 1991). The distance from the tip of the cytoplasmic filaments to the terminal ring in the nuclear basket is roughly 200 nm (Fig. 6). Cytoplasmic filaments and nuclear basket are the sites of docking of import and export substrates, respectively (Kiseleva et al., 1997; Richardson et al., 1988).

Vertebrates NPCs are large 125 KDa structures with an outer diameter of approximately 120 nm. They are estimated to contain around 1000 proteins with 6-8 copies of some 50-100 different proteins, collectively known as the nucleoporins (Bastos et al., 1995). Many of these proteins have been identified, and their functions and localization in the NPC have been characterized. Most were found on either the cytoplasmic filaments or in the nuclear basket. Only few were associated with the basic framework (Nigg, 1997). Mutations in several nucleoporins revealed defects in both nuclear import and export, indicating that these processes are coupled (Davis, 1995; Doye and Hurt, 1995). Characteristic features of the nucleoporins include the presence of FXFG or GLFG (FG repeats), coiled-coil domains and *O*-linked *N*-acetylglucosamine modifications. The FG repeats have been shown to interact *in vitro* with transport factors and are speculated to mediate their translocation across the NPC through docking and undocking reactions with the nucleoporins (Iovine et al., 1995; Radu et al., 1995; Rexach and Blobel, 1995; Stutz et al., 1996). The nucleoporin coiled-coil domains are speculated to promote the assembly of nucleoporin sub-complexes, and the function of the *O*-linked *N*-acetylglucosamine modifications remains unknown (Nigg, 1997).

Two nucleoporins (Nup98, Nup214) have been identified as fusion partners in chromosomal translocations associated with myeloid leukemia (Borrow et al., 1996a; Kraemer et al., 1994; Nakamura et al., 1996b; Raza-Egilmez et al., 1998); however, the molecular mechanisms by which oncogenesis occurs remain unclear. In the case of the Nup98-HOXA9 chimera, the FG repeats are fused to the PBX-heterodimerization domain and the homeodomain of HOXA9. The fusion protein has been shown to bind through its homeodomain and to activate transcription through recruitment of the coactivator CBP by the FG repeats (Kasper et al., 1999). This transactivation function could be mediated as well by the FG repeats of Nup 153 or Nup214, suggesting a direct function for the FG repeats in transformation.

4.1.2 *Nucleocytoplasmic transport: signals and receptors*

Nucleocytoplasmic transport is mediated by the presence of nuclear localization signals (NLS) or nuclear export signals (NES) in the substrates or "cargo" that are recognized by NLS and NES receptors, respectively. There are two forms of classical NLS. The monopartite NLS is a stretch of 5 to 7 basic residues that is best illustrated by the NLS of the SV40 large T antigen, PKKKRKV (Kalderon et al., 1984a; Kalderon et al., 1984b). The bipartite NLS, as in the nucleoplasmin protein, is composed of two basic amino acid clusters separated by a linker region of 10 residues $(K/R)_2 10$ aa $(K/R)_{3/5}$ (Robbins et al., 1991). These NLS types are found in a wide range of nuclear proteins; however, not all basic stretches function as NLS. An NLS is defined by two criteria: mutation or deletion of the NLS leads to cytoplasmic accumulation of a nuclear protein; and when fused to a non-nuclear protein, the NLS directs the protein to the nucleus

(Silver, 1991). The position of the NLS and its context in the protein are important factors regulating its function. For example, the SV40 T antigen NLS does not function if buried in the hydrophobic domain of the pyruvate kinase; it needs to be exposed on the surface of the protein to interact with the transport machinery (Silver, 1991). Conformational changes in a protein can mask or uncover an NLS. This is seen for instance in the glucocorticoid receptor, where ligand binding to the hormone binding pocket changes the conformation of the receptor and exposes its NLS (Picard and Yamamoto, 1987); similarly phosphorylation or dephosphorylation of a protein have been reported to regulate their NLSs activity (Beals et al., 1997; Beg et al., 1992; Henkel et al., 1992).

Non-classical NLSs have also been identified that interact with import mediators that do not recognize the classical type of NLSs. Proteins with classical NLS are transported to the nucleus by the importin α/β complex (Adam and Gerace, 1991; Adam et al., 1989). While importin α is an adapter that recognizes the NLS, importin β docks the NLS-importin α complex to the NPC (Gorlich et al., 1995a; Gorlich et al., 1995b). Two other adapters have been identified in vertebrates that function with importin β. These include snurportin 1 that recognize U snRNAs (Huber et al., 1998) and replication A interacting protein α (RIPα) that binds to the 70 KDa subunit of the replication protein A (Jullien et al., 1999). All three adapters interact with importin β through a domain known as importin β binding domain or IBB. The IBB in importin α is a 41 basic-rich aa region that contains a motif resembling a monopartite NLS (Gorlich et al., 1996a; Weis et al., 1996). The presence of such a motif suggests that some cargo can bind directly to importin β This is the case for the HIV-1 Rev and Tat proteins (Truant and Cullen,

1999), HTLV Rex (Palmeri and Malim, 1999), cyclin B1 (Moore et al., 1999) and at least some of the ribosomal proteins (Jakel and Gorlich, 1998). These proteins possess extended and very basic-rich NLSs that do not require an adapter for recognition. Besides functioning on its own or with an adaptor such as importin α, importin β has been shown to collaborate with other NLS receptors. For example, the nuclear import of histone H1 requires the heterodimer importin β/importin 7 (Jakel et al., 1999). In addition, to binding cargo, importin β also interacts with FG repeat-containing nucleoporins (Nakielny et al., 1999; Wu et al., 1995) and with the GTPase RAN (see below). Once in the nucleus, the adapter/receptor dissociates from the import cargo and recycles back to the cytoplasm to repeat the cycle with other NLS-containing proteins.

The first NESs discovered are those of the HIV-1 Rev (Fischer et al., 1995) and the protein kinase inhibitor (PKI) (Wen et al., 1994). These NESs are composed of short leucine-rich aa stretches as in the consensus $L(X)_{1-4}L(X)_{2-3}LXL$, and are referred to as the leucine-rich or Rev-like NESs. Comparison of different Rev-like NESs revealed that the presence of a leucine is not essential and that hydrophobic residues including isoleucine, valine and methionine can substitute for the leucine. Similar to nuclear import, nuclear export is saturable, thus involving a receptor for the NES, and is energy-dependent (Fischer et al., 1995). The Rev-like NES receptor known as CRM-1/exportin1 is homologous to importin β (Fornerod et al., 1997a; Fornerod et al., 1997b; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). CRM-1/exportin1 was initially identified in *S.pombe* as a protein involved in chromosomal maintenance, thus its name (Adachi and Yanagida, 1989). Its export function is sensitive to leptomycin B (LMB), an anti-fungal antibiotic shown to induce cell cycle arrest (Nishi et al., 1994; Yoshida et al.,

1990). LMB prevents the export of 5S RNA and U snRNA but not that of tRNA or mRNA, suggesting the presence of other NES-receptors (Fornerod et al., 1997a). The second NES-receptor identified is exportin-t, involved in the export of tRNA (Kutay et al., 1998). The export of the nuclear import adapters importin α and snurportin-1 is mediated respectively by CAS (*C*ellular *A*poptosis *S*uceptibility) (Kutay et al., 1997) and by CRM-1/exportin1. These proteins bind preferentially to the free form of the adapter (i.e. after it has released its cargo) (Paraskeva et al., 1999). In contrast to adapters, the transport receptors are thought to be recycled back to their original cellular compartment in a receptor-independent fashion via direct interactions with the NPC (Mattaj and Englmeier, 1998).

Nucleocytoplasmic transport can be divided into three steps: energy-independent docking of the receptor/cargo to the NPC, energy-dependent translocation across the NPC, and release of the cargo from the receptor. The directionality of the transport and its energy requirements are derived from the presence of a GTPase "RAN" in the nucleus, and a GTP concentration gradient across the NE (Gorlich et al., 1996b; Izaurralde et al., 1997; Melchior et al., 1993; Moore and Blobel, 1993). RAN stands for RAS-related nuclear protein (Drivas et al., 1990). Unlike other GTPases, RAN is not prenylated at its C-terminus and thus does not bind to cell membranes. Its activity is maximal in the presence of cofactors such as RAN GAP1 (RAN GTPase activating protein 1) (Bischoff et al., 1994) and RAN BP1 (Ran binding protein 1) (Bischoff et al., 1995), both cytoplasmically localized. The conversion of RAN-GDP to RAN-GTP is mediated by the nuclear protein RAN guanine exchange factor (RAN GEF) (Bischoff and Ponstingl, 1991a; Bischoff and Ponstingl, 1991b). The presence of RAN GEF in the

nucleus and of the RAN cofactors in the cytoplasm result in RAN-GTP predominantly in the nucleus and RAN-GDP in the cytoplasm. In the case of nuclear import, interaction with RAN-GTP is required for the last transport step (Gorlich et al., 1996b); it modulates the affinity of the receptor for its cargo resulting in the release of the cargo from the receptor and of the receptor from the nucleoporins (Rexach and Blobel, 1995). In contrast, nuclear export receptors require RAN-GTP interaction for substrate binding (Fornerod et al., 1997a; Kutay et al., 1997; Kutay et al., 1998). Upon interaction with RAN-GTP, the export receptor/cargo/RAN-GTP complex translocates into the cytoplasm, where RAN-GTP hydrolysis into RAN-GDP takes place and results in the dissociation of the export cargo from its receptor (Fornerod et al., 1997a; Kutay et al., 1997; Kutay et al., 1998). The final step to restore the RAN-GTP gradient would then be the import of RAN-GDP back into the nucleus. RAN does not possess an NLS (Ren et al., 1993), and its import is mediated by a 14 KDa protein known as p10/NTF2 (Paschal et al., 1996; Ribbeck et al., 1998). It has been demonstrated that p10/NTF2 interacts with different FG-containing nucleoporins located all across the NPC (Nehrbass and Blobel, 1996; Paschal and Gerace, 1995); therefore it is believed to translocate across the NPC through docking and undocking reactions with the nucleoporins, similar to importin β.

4.2 Regulation of PBX nuclear localization in limb development

The subcellular localization of PBX/EXD proteins was shown to be under the control of MEIS/PREP1/HTH family members (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Rieckhof et al., 1997; Saleh et al., 2000a). In the absence of HTH, EXD is in the cytoplasm. In the presence of HTH/MEIS/PREP1, EXD/PBX translocates into the

nucleus, a process that requires direct interaction between MEIS family members and PBC proteins. Mapping analysis indicated that regions PBC-A in PBX/EXD and region HM1 in MEIS family members are the domains involved in such interactions (Abu-Shaar et al., 1999; Jaw et al., 2000). What is the mechanism behind this regulation? It was recently demonstrated that EXD/PBX accumulates in the nucleus as a result of the inhibition of its LMB-sensitive nuclear export by HTH/PREP1 (Abu-Shaar et al., 1999; Berthelsen et al., 1999). Both studies characterized a non-consensus NES in EXD and PBX; however, different protein domains were proposed to harbor this putative NES. the NES localized to region PBC-B in EXD while it was mapped to PBC-A in PBX. Therefore, Berthelsen *et al.* proposed a direct competition between PREP1 and the nuclear export factor (both bind to the same domain in PBX, PBC-A). On the other hand, Abu Shaar *et al.* implied a conformational change in EXD, induced by HTH binding to PBC-A, that alters its interaction in PBC-B with the nuclear export factor (Abu-Shaar et al., 1999; Berthelsen et al., 1999). These observations suggest that PBX/EXD may not have an NES of their own. They may translocate to the cytoplasm through the binding of an intermediary protein or adapter that itself possesses an NES. The binding of this adapter to PBX/EXD would be the step regulated by MEIS/HTH.

The second question to be asked is how HTH/MEIS/PREP1 regulates the nuclear import of EXD/PBX. There are different mechanisms by which EXD/PBX can get to the nucleus. First, PBX could simply diffuse into the nucleus, given that its size (M_r=50 KDa) is smaller than the 60 KDa cut-off for passive diffusion through the NPC. Second, PBX could piggyback on MEIS/PREP1 and be carried into the nucleus. Third, PBX could possess its own NLS; however, it requires binding with MEIS/PREP1 to unmask its

NLS. We provide evidence supporting the third mechanism in chapter 2 (Saleh et al., 2000a).

Control of the nuclear localization of EXD/PBX has been shown to be essential for proper limb development both in *Drosophila* and vertebrates. In *Drosophila* leg primordia, both *Hth* expression and nuclear EXD are restricted to proximal cells. In distal cells, *Hth* is not expressed and EXD is cytoplasmic (Casares and Mann, 1998). *Hth* expression is repressed in the distal cells by the products of the homeobox-containing genes *distalless* (*dll*) and *dachshund* (*dac*), which are under the control of HEDGEHOG (HH) signaling and its downstream DPP (DECAPENTAPLEGIC) and WG (WINGLESS) pathways (Gonzalez-Crespo et al., 1998).

In the vertebrate limb, two major organizing centers exist at the distal tip and are responsible for the regulation of appendage outgrowth. The first is derived from dorsal-ventral interface of the limb epithelium and is known as the apical ectodermal ridge (AER). The second is the "progress zone" formed by the mesenchyme that underlies the AER. Interactions between the AER and the progress zone determine the extent of limb growth. The AER produces fibroblast growth factors (FGF4 and FGF8) that signal the progress zone mesenchymal cells to proliferate, and in turn factors produced by the progress zone, which include SHH (SONIC HEGEHOG), BMPs (bone morphogenetic proteins; DPP homologues) and the downstream BMPs antagonists NOGGIN and GREMLIN, maintain the AER (for review, seeMartin, 1998; Merino et al., 1999; Zuniga et al., 1999). The first evidence suggesting a conserved role of MEIS/nuclear PBX in vertebrate limb development came from the observation that, in the mouse limb, PBX is nuclear in proximal cells and is cytoplasmic in distal cells. This distribution presents an

evolutionary parallel to EXD nuclear distribution along the fly limb primordia (Gonzalez-Crespo et al., 1998). Further proof came from two recent studies by Capdevila *et al.* and Mercader *et al.* that examined the effects of ectopic overexpression of *Meis1* and *Meis2* genes in developing chick limb buds and *Drosophila* wing and leg imaginal discs. Results from these studies show that the presence of MEIS directs nuclear localization of PBX in the distal limb and leads to distal truncations or proximalization of distal structures (Capdevila et al., 1999; Mercader et al., 1999). Truncated appendages are also observed in *Shh* null mice (Chiang et al., 1996), suggesting that repression of *Meis* expression in the vertebrate distal limb could be under the regulation of the AER SHH signaling, as is the case in *Drosophila*. In parallel to the role of DPP in restricting *Hth* expression, vertebrate BMPs were also shown to restrict the proximal expression of *Meis* (Capdevila et al., 1999). Implantation of BMP-2-soaked beads in the proximal limb resulted in repression of *Meis2* expression. Whether this regulation involves vertebrate *dll*-homologues such as the *dlx* genes has not yet been investigated. In summary, control of the subcellular localization of PBX proteins is required to subdivide the limb into proximal and distal parts. In addition, the mechanism of its regulation by the MEIS family and upstream distal signaling cues from the AER and the progress zone has been evolutionary conserved between insects and vertebrates.

5. REGULATION OF TRANSCRIPTION BY THE HOX•PBX COMPLEX

5.1 Chromatin-modifying proteins in association with HOX•PBX complexes

It has been well established that eukaryotic transcription is regulated by chromatin structure. Packaging of genes into chromatin represses basal transcription. Transcriptional activators function, at least in part, to override chromatin-mediated repression. Chromatin-modifying machines include the chromatin remodeling factors and the HATs and HDACs. Interactions of these complexes with transcription factors and the general transcription machinery are key events in transcriptional regulation.

5.1.1 *Chromatin-remodeling machines*

The first chromatin remodeling factors of the SWI/SNF complex were identified genetically in yeast as regulators of transcription (Sudarsanam and Winston, 2000). SWI/SNF is a 2 MDa complex that comprises an estimated 11 polypeptides conserved from yeast to man (Wang et al., 1996a; Wang et al., 1996b). Evidence supporting SWI/SNF function in transcriptional regulation came from *in vitro* studies that demonstrated its ability to remodel chromatin by locally disrupting the association of histones with DNA, permitting access of transcription factors to their binding sites (Schnitzler et al., 1998). Recent studies have shown that SWI/SNF could be targeted to specific promoters by transcriptional activators in an activation-domain-dependent manner, resulting in stimulation of transcription from nucleosome arrays (Neely et al., 1999). Surprisingly, other studies have revealed that SWI/SNF is required for transcriptional repression. For instance, the nuclear receptor corepressor N-coR was recently shown to copurify with SWI/SNF-related chromatin remodeling complexes (Underhill et al., 2000). Similarly, the retinoblastoma protein (pRB) has been shown to recruit SWI/SNF to repress E2F1 transcriptional activity (Trouche et al., 1997). In

addition, pRB-dependent repression of the *c-fos* gene is mediated by a member of the SWI/SNF complex (Murphy et al., 1999). SWI/SNF is the founder of a growing family of chromatin remodeling machines. These include the yeast RSC complex, *Drosophila* NURF, CHRAC, ACF and BRM complexes, mammalian BRG1- or hBRM-associated complexes, and NURD. A common feature of all these complexes is that they contain a motor protein with a DNA-dependent ATPase activity that is speculated to translocate on DNA and disrupt histone-DNA interactions (reviewed in Pazin and Kadonaga, 1997).

5.1.2 *Chromatin-remodeling machines in relation to HOX function*

Hox gene expression and function have been shown to be regulated by chromatin remodeling machines. As mentioned above, the repressed and activated states of *Hox* genes are maintained through the action of PcG and TrxG proteins. Some of the PcG and TrxG characterized so far are members of chromatin remodeling machines. For example, BRAHMA (BRM), a TrxG component, is the *Drosophila* homologue of the yeast SWI2/SNF2 chromatin remodeling motor protein (Tamkun et al., 1992); GAGA, the product of the *Trx-like* gene, recruits NURF (nucleosome remodeling factor) to displace nucleosomes (Tsukiyama and Wu, 1995). In addition, repression of *Hox* genes by the gap gene product Hunchback (HB) and subsequently by PcG proteins has been demonstrated to be mediated by direct recruitment of dMi2 by HB (Kehle et al., 1998). dMi2 is the *Drosophila* homologue of the ATPase subunit -Mi2- of the nucleosome remodeling and deacetylation complex (NURD) (Wade et al., 1998; Xue et al., 1998; Zhang et al., 1998a).

HOX proteins on the other hand have been shown to interact with NURD to regulate the transcription of downstream targets. Genetic evidence from *C. elegans* revealed that EGL-27, a homologue of the NURD component MTA1 (metastasis-associated factor), modulates the transcriptional functions of two HOX proteins , LIN-39 and MAB-5, during pattern formation (Solari et al., 1999) (Ch'ng and Kenyon, 1999; Herman et al., 1999). These data suggest that HOX proteins mediate their functions by modulating the chromatin structure through recruitment of SWI/SNF-like complexes.

5.1.3 *HATs/HDACs*

It is widely believed that histone acetylation is generally associated with active transcription while histone deacetylation is associated with repression (reviewed in Grunstein, 1997; Struhl, 1998). Acetylation occurs at lysine residues in the N-terminal tails of the histones, thereby neutralizing their positive charge and decreasing their affinity for DNA (Hong et al., 1993). As a consequence, histone acetylation results in a conformational change in chromatin (Norton et al., 1989), thereby increasing the accessibility of transcriptional regulators for their binding sites in nucleosomal templates (Lee et al., 1993; Vettese-Dadey et al., 1996). Discovery of proteins with intrinsic HAT and HDAC activities greatly improved the understanding of the link between histone modifications and transcription. Some of these proteins are components of the RNA polymerase II (POLII) general transcription machinery, transcriptional activators or repressors, or transcriptional coregulators.

According to their function in transcription, the mammalian HATs are subdivided into five families. The prototype family that includes GCN5 (Smith et al., 1998) and

P/CAF (*p*300/*C*BP-*a*ssociated *f*actor) (Yang et al., 1996b), homologues of the yeast activator GCN5 (Brownell et al., 1996), the CBP/p300 coactivator family (Bannister and Kouzarides, 1996; Ogryzko et al., 1996), the TAF250 (TFIID-associated factor) family (Mizzen et al., 1996), the nuclear receptor coactivators SRC1 (steroid receptor coactivator) and ACTR (activator of thyroid and retinoid receptors) family (Chen et al., 1997a; Spencer et al., 1997) and the MYST family that includes Tip60 and MOZ (Borrow et al., 1996b; Hilfiker et al., 1997; Kamine et al., 1996). In addition to acetylating histones, P/CAF and CBP/p300 have also been shown to acetylate non-histone proteins, many of which are involved in the regulation of transcription. These include TFIIF, TFIIEβ, p53, E2F1, EKLF, TCF, GATA1, HMGI(Y) and ACTR (reviewed in Kouzarides, 2000).

The mammalian HDACs form three classes based on homology to their yeast counterparts. Class I includes HDAC1, HDAC2, HDAC3 and HDAC8, homologues of the yeast repressor RPD3. Class II includes HDAC4, HDAC5, HDAC6 and HDAC7, homologues of yHDA1. Class III has been recently identified and is composed of one NAD-dependent HDAC with homology to ySIR2 protein (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000). HDACs from class I and class II but not from class III can be potently inhibited by the drugs Trichostatin A (TSA) and trapoxin (Yoshida et al., 1995). HDAC1 and HDAC2 are found in two distinct large multi-protein histone deacetylase complexes -mSIN3A/B•HDAC and NURD- that were purified from mammalian cells and shown to be important to target HDAC activity to nucleosomes (Wade et al., 1998; Xue et al., 1998; Zhang et al., 1997; Zhang et al., 1998a; Zhang et al., 1998b). While mSIN3A/B•HDAC complex has been reported to be recruited by multiple transcriptional

regulators to repress transcription (Hassig et al., 1997; Kadosh and Struhl, 1997; Laherty et al., 1997; Nagy et al., 1997; Sun and Hampsey, 1999), NURD was recently demonstrated to be recruited to methylated DNA and may thus function in gene silencing by DNA methylation (Ng et al., 1999; Wade et al., 1999; Zhang et al., 1999).

5.1.4 *HATs/HDACs in association with HOX•PBX proteins*

Various pieces of evidence relate *Hox* gene regulation and HOX•PBX protein functions to HATs and HDACs. Indirect evidence came from the phenotypes of *cbp/p300* null embryos in the mouse, mutations in *Cbp* in *Drosophila* and *Cbp* loss of function in man (Rubinstein-Taybi syndrome), in which multiple developmental defects (defects in pattern formation and cell proliferation) are reminiscent of those caused by mutations in *Hox* genes (Akimaru et al., 1997; Petrij et al., 1995; Yao et al., 1998). Genetic studies from *Drosophila* revealed an interaction between *Dfd*, the fly orthologue of *Hoxd4*, and *Nejire* also known as *dCbp* (Florence and McGinnis, 1998), providing a link between HOX function and CBP. A direct interaction between a HOX protein and CBP was recently shown for HOXB7 (Chariot A, 1999).

In addition to modulating HOX functions, CBP has been shown to alter *Hox* gene expression. In response to WG signaling, *Drosophila* CBP was shown to repress *lab* expression and the *Ubx* enhancer function in the visceral endoderm and mesoderm, respectively (Waltzer and Bienz, 1998). In contrast, CBP activated these *Hox* genes in response to DPP signaling (Waltzer and Bienz, 1999). Regulation of *Hox* expression by CBP implied an interaction between HOX proteins and CBP considering the various

auto- and crossregulatory loops that are involved in the regulation of *Hox* expression (see above).

Prior to our work (chapter 3), recruitment of HDACs by HOX•PBX complexes was not demonstrated; however, PBX was shown to interact with the corepressor N-coR in a PDX•PBX complex (Asahara et al., 1999). In the case of nuclear receptors, N-coR mediates transcriptional repression *via* the recruitment of the mSIN3A/B•HDAC complex (Nagy et al., 1997) or *via* interaction with HDAC7 (Kao et al., 2000). This suggested that PBX may recruit HDACs *via* the N-coR corepressor. We demonstrate that, in a HOX•PBX complex, PBX recruits the mSIN3B•HDAC complex to repress transcription of HOX downstream targets (chapter 3).

5.2 *HOX proteins and the general transcription machinery*

RNA polymerase II-driven transcription depends on the formation of a preinitiation complex (PIC) and the subsequent recruitment of the POLII holoenzyme (Buratowski, 2000; Cox et al., 1998; Severinov, 2000). The initial step in the assembly of the PIC is the recognition of the promoter by TFIID (Horikoshi et al., 1989; Horikoshi et al., 1990; Starr and Hawley, 1991). TFIID includes the TATA-binding protein (TBP) and a complex of at least seven polypeptides termed TBP-associated factors or TAFs of different molecular weights (250, 150, 110, 80, 60, 40 and 30 KDa) (Andel et al., 1999; Poon et al., 1995; Pugh and Tjian, 1991; Tanese et al., 1991; Zhou and Berk, 1995). Among other functions, TAFs have been suggested to mediate interactions between transcriptional activators/repressors and the POLII holoenzyme, and to contribute to the stability of binding of the PIC (for review, see Hahn, 1998). TFIID binding to the TATA

box facilitates the sequential recruitment of the general transcription factors (GTFs) TFIIB and TFIIF, followed by that of the POLII holoenzyme and then that of TFIIE and TFIIH (Cox et al., 1998). The POLII holoenzyme has been recently shown to include, in addition to the core POLII (the twelve-subunit enzyme), a mediator complex (MED)/SRB (for review, see Chang and Jaehning, 1997). MED/SRB components act as transcriptional coregulators that mediate, in most reported cases, activation of transcription. However, some forms of MED complexes have been described as corepressors (Song and Carlson, 1998; Sun et al., 1998). MED/SRB proteins associate with the C-terminal domain (CTD) of the largest subunit of POLII, explaining the fact that they were identified in yeast as suppressors of the lethality caused by mutations in the POLII CTD (Thompson et al., 1993). *In vitro* transcription assays demonstrated that the POLII holoenzyme, in combination with TBP, respond to activator proteins even in the absence of TAFs. This suggested that the mediator components could act as targets of transcriptional activators to stimulate basal transcription initiation (for review, see Struhl, 1996). In mammals, several MED complexes have been isolated through their physical association with different transcription factors. These include TRAP complex (in association with the thyroid receptor; Ito et al., 1999), DRIP complex (vitamin D3 receptor; Rachez et al., 1999), CRSP1 (SP1; Ryu et al., 1999), ARC (SREBP-1a, VP16, NF-κB; Naar et al., 1998), and other complexes recruited by p53 (Ito et al., 1999) and E1A (Boyer et al., 1999).

Very little is known about the association of HOX proteins with components of the general transcription machinery. However, two reports have suggested a competition between the MSX1 (Zhang et al., 1996a) and EN (engrailed) (Ohkuma et al., 1990)

homeoproteins and TFIID, for binding to the TATA box. Such a competition was shown to result in transcriptional repression by these homeoproteins. More recently, a genetic study in *Drosophila* has provided evidence of an interaction between a HOX protein, SCR, and the MED/SRB complex (Boube et al., 2000). This study provides an important link connecting HOX•PBX, bound to target enhancers, to the general transcription machinery.

The cumulative data from all the studies listed above greatly clarify our view on how HOX•PBX regulate transcription. The HOX•PBX complex may modify the chromatin around the promoter of its target gene through recruitment of chromatin modifying factors, and associate with the general transcription machinery *via* direct binding with the mediator complex. It would be interesting to characterize all such interactions for one particular HOX protein *in vivo* and study the kinetics of the recruitment of these proteins to regulate the expression of a known downstream target during development (see discussion).

6. PERSPECTIVE

HOX proteins achieve functional specificity through interactions with DNA-binding partners of the PBX and MEIS/PREP1 families. HOX•PBX•MEIS/PREP1 heterotrimeric complexes form *in vivo* on enhancers of HOX downstream targets, and regulate their functions. The nuclear availability of HOX cofactors is hence essential for the transcriptional regulation mediated by HOX proteins. Interestingly, MEIS/PREP1 proteins regulate PBX nuclear localization. The work presented in this thesis addresses the mechanisms behind such a regulation (chapter 2). We show that MEIS/PREP1 induce

a conformational change in PBX that is necessary for the unmasking of its buried NLS, and hence for its nuclear localization. This thesis also explores the mechanisms of transcriptional regulation by HOX•PBX complexes (chapter 3). HOX•PBX represses transcription in an HDAC-dependent manner. PBX recruits a corepressor complex consisting of N-coR, mSIN3B and class I HDACs *via* its N-terminal repression domains. In response to PKA signaling or as a result of cell aggregation, HOX•PBX are switched into transcriptional activators. Activation is achieved through the recruitment of the HAT/coactivator CBP by the HOX protein's activation domain. The net transcriptional function of HOX•PBX complexes is hence determined by a balance of coactivators *versus* corepressors, associated with HOX and PBX, respectively, in response to cell signaling. Conclusions drawn from this work improve our understanding of HOX protein functions in the patterning of the animal embryo. In addition, it lays the ground for future experiments aimed to address the role of MEIS in the transcriptional regulation mediated by HOX•PBX•MEIS complexes, and the differential response of such complexes to variable signaling pathways.

CHAPTER II

A Conformational Change In PBX1A Is Necessary For Its Nuclear Localization

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1. ABSTRACT

The fly homeodomain (HD) protein EXTRADENTICLE (EXD) is dependent on a second HD protein, HOMOTHORAX (HTH) for nuclear localization. We show here that in insect cells the mammalian homolog of EXD, PBX1A, shows a similar dependence on the HTH homologs MEIS1, 2 and 3, and the MEIS-like protein PREP1. Paradoxically, removal of residues N-terminal to the PBX1A HD abolishes interactions with MEIS/PREP but allows nuclear accumulation of PBX1A. We use deletion mapping and fusion to green fluorescent protein (GFP) to map two cooperative nuclear localization signals (NLS) in the PBX HD. The results of DNA-binding assays and pull-down experiments are consistent with a model whereby the PBX N-terminus binds to the HD and masks the two NLS. In support of the model, a mutation in the PBX HD that disrupts contact to the N-terminus leads to constitutive nuclear localization. The HD mutation also increases sensitivity to protease digestion, consistent with a change in conformation. We propose that MEIS family proteins induce a conformational change in PBX that unmasks the NLS leading to nuclear localization and increased DNA-binding activity. Consistent with this, PBX1 is nuclear only where *Meis1* is expressed in the mouse limb bud.

2. INTRODUCTION

In mammals, *Hox* genes constitute a family of 39 members arranged in 4 clusters. These clusters are believed to have duplicated from the same ancestral cluster that gave rise to the *Drosophila Hox* genes (reviewed in McGinnis and Krumlauf, 1992). A role in the specification of antero-posterior identity is conserved across species. HOX proteins are HD-containing transcription factors (reviewed in Graba et al., 1997). The PBC family of HD proteins comprises mammalian PBX, *Drosophila* EXD and *Caenorhabditis elegans* ceh-20 (Monica et al., 1991). High-affinity DNA-binding is achieved when HOX proteins are heterodimerized with PBC-family members (Mann and Chan, 1996). Mammalian MEIS1 (Moskow et al., 1995) has also been shown to independently dimerize with HOX proteins (Shen et al., 1997b) and with PBX (Chang et al., 1997b). Recently, trimeric complexes comprising HOX, PBX and MEIS partners have been reported (Jacobs et al., 1999; Schnabel et al., 2000; Shanmugam et al., 1999; Shen et al., 1999; Swift et al., 1998). A MEIS-related protein PREP1 (Berthelsen et al., 1998b), also known as PKNOX1 (Chen et al., 1997b), can additionally form a dimer with PBX as well as a trimeric complex with HOX and PBX partners (Berthelsen et al., 1998a; Ferretti et al., 2000; Knoepfler et al., 1997; Penkov et al., 2000). Physical interaction between PBX and either MEIS1 or PREP1 is dependent on residues located towards the N-terminus of all three proteins. Thus, the E2A-PBX oncoprotein, lacking residues 1 to 89 of PBX1, is unable to dimerize with either MEIS1 or PREP1 (Chang et al., 1997a; Knoepfler et al., 1997).

In *Drosophila*, the MEIS homolog HTH directs the nuclear localization of EXD (Aspland and White, 1997; Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997).

This partly explains the observation that the *Hth*$^{-/-}$ phenotype (Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997) is similar to that of *exd*$^{-/-}$ (Peifer and Wieschaus, 1990), both presenting homeotic transformations without altering *Hox* gene expression. In an *Hth*$^{-/-}$ background EXD is cytoplasmic. EXD is also cytoplasmic in the blastoderm embryo when *Hth* is not yet expressed. Mammalian MEIS1 can substitute for the fly HTH protein for successful translocation of EXD to the nucleus (Rieckhof et al., 1997), strongly suggesting that similar mechanisms should operate in the subcellular control of PBX family members.

Hth is repressed in the distal cells of the leg imaginal disc and hence EXD is localized in the cytoplasm (Gonzalez-Crespo et al., 1998). Similarly, PBX1A is cytoplasmic in the distal cells of the mouse limb primordium (Gonzalez-Crespo et al., 1998). This further suggests that in mammals, MEIS family members may play a regulatory function in PBX1A subcellular localization.

Recently, two studies have examined the control of PBX nuclear localization and showed that one role of HTH and PREP1 proteins in this process is the inhibition of EXD/PBX nuclear export (Abu-Shaar et al., 1999; Berthelsen et al., 1999). However, treatment with the nuclear export inhibitor LMB results in only partial nuclear retention of endogenous EXD (Abu-Shaar et al., 1999). This suggests that, in addition to nuclear export, other mechanisms controlled by MEIS/PREP1 may be involved in the regulation of EXD/PBX nuclear localization.

Two additional mechanisms may render PBX dependent on MEIS family members for nuclear entry. First, PBX may lack a nuclear localization signal (NLS) and therefore gain entry to the nucleus by using MEIS as a carrier. Second, interaction with MEIS may

be required to expose an otherwise masked NLS in PBX1A. Unmasking of the NLS would then lead to an active, energy-consuming translocation to the nucleus (precedents reviewed in Mattaj and Englmeier, 1998; Nigg, 1997).

In this report, we have investigated these additional mechanisms. We show that MEIS1, 2 and 3 plus PREP1 all direct the nuclear localization of PBX1A in insect cells. Removal of the entire N-terminus, including the MEIS/PREP interaction domain, renders PBX1A constitutively nuclear due to the action of two cooperative NLS within the HD. Thus, PBX1A is not simply dependent on MEIS family members to furnish NLS activity. We show direct physical interaction between the PBX1 N-terminus and the HD, and suggest that these intramolecular contacts mask both NLS. Accordingly, MEIS/PREP family members would bind the PBX N-terminus thereby inducing a conformational change that exposes the NLS in the HD. In support of this model, an HD mutant that disrupts contact to the N-terminus renders PBX1A constitutively nuclear. Consistent with a role in the nuclear localization of PBX proteins, PBX is nuclear in the *Meis1* expression domain within the mouse limb bud.

3. EXPERIMENTAL PROCEDURES

3.1 Expression vectors

All the cDNAs used in this study were subcloned in both pCS2+, a mammalian expression vector bearing the CMV promoter, and pPACPL, a Drosophila expression vector with a constitutively active β actin promoter. PBX1A-HA was derived in part from the human EPΔGKFQ described previously (Green et al., 1998) with the HA tag sequence (encoding three HA epitopes: YPYDVPDYA) replacing residues 296 to 308 of PBX1A. Δ172-219-HA, Δ172-254-HA and Δ172-295-HA were constructed by PCR amplification. Δ284-293 was constructed by PCR overlap extension. Both Δ1-89-HA and Δ1-232-HA were constructed by PCR amplification and insertion in pRc-CMV (Invitrogen) 3' to the HA tag. The HD-GFP fusion vector as well as Δ285-296-GFP were constructed by PCR amplification and insertion in frame of PBX1A region 219-295 and 219-286, respectively, 3' to GFP in a CMV-GFP vector. R235L/K236E and N51S were constructed by site-directed mutagenesis using Quik Change^{TM} (Stratagene). GST-HD fusion was constructed by inserting PBX1A HD (amino acids 233-295) in the prokaryotic expression vector pGEX3X (Pharmacia) 3' to GST. All mutations generated by PCR or site-directed mutagenesis were verified by sequencing. E2A-PBX1 E28R was a generous gift of M. Kamps and is described elsewhere (Calvo et al., 1999). PBX E28R was generated by cloning the StuI-NcoI fragment from E2A-PBX1 E28R into PBX1A in pCS2+.

3.2 Transfections and LMB treatment

Cos-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Cells were plated at a density of 300,000 cells in 35 mm tissue culture plates containing 3 uncoated glass coverslips. The cells were allowed to attach for at least 16 h and were transfected either by electroporation with 10 μg recombinant DNA or using the calcium phosphate co-precipitation method with recombinant DNA concentrations ranging from 100 ng to 2 μg plus carrier DNA to a total of 15 μg. The precipitate was removed 24 h post-transfection and cells were incubated for a further 24 h. Results with both transfection methods were comparable. Drosophila Schneider's (S2) cells were grown in Schneider's medium supplemented with 10% fetal calf serum (FCS). Cells were plated at a starting density of 3 million cells in 35 mm tissue culture plates containing uncoated glass coverslips. The transfection protocol was the same as for Cos-7 cells except that only the calcium phosphate co-precipitation method was used with a total of 20 μg recombinant DNA. LMB treatment was done as follows: S2 cells were transfected as above followed by LMB treatment (100 nM or 250 nM) for 3 to 5 h prior to the fixation of the cells.

3.3 Histological techniques

Transfected cells were washed with phosphate-buffered saline (PBS) and fixed with 3% paraformaldehyde for 5 min at room temperature. For the detection of either GFP or GFP fusion proteins, the cells were washed with PBS and mounted at this stage on microscopic slides using immunoflor mounting media (Sigma). Otherwise, the cells were

washed with PBS and permeabilized with 0.2% Triton X-100 (in PBS) for 2 min at room temperature, to allow better penetrance of antibodies. After permeabilization, the cells were washed with PBS and blocked with fetal calf serum to saturate non-specific binding sites for 1 h in a humid chamber at room temperature, followed by incubation with mouse anti-HA monoclonal antibody (Berkeley Antibody Company) for 2 h at room temperature. The cells were subsequently washed 3 times with cold PBT (PBS with 0.2% Tween 20) then incubated with rhodamine-linked goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature in the dark. Lastly the cells were washed 3 times with PBT before being mounted on microscopic slides using immunoflor mounting media. Histological controls included staining untransfected cells and using anti-flag monoclonal antibody as a non-specific antibody against the HA tag. The cells were analyzed with a Nikon ECLIPSE E800 fluorescent microscope and with a Biorad MRC 600 confocal microscope. The extent of nuclear localization was scored following the scheme of Ylikomi et al. (Ylikomi et al., 1992).

3.4 Electrophoretic mobility shift assay

EMSA was conducted as described previously (Phelan et al., 1994) except that the final buffer conditions were 10 mM Tris (pH7.5), 75 mM NaCl, 1mM dithiothreitol, 1mM EDTA, 5.4 μg bovine serum albumin, 12.7% glycerol. No poly(dI-dC) was included in the reactions.

3.5 GST-pull down assay

Pull down experiments were performed with *in vitro* translated and ^{35}S-labeled PBX1A 1- 232 or luciferase incubated for 90 min at 4°C in NET-N buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 8.0), 1.0% Triton X-100, 1μM Leupeptin, 1μM Pepstatin, 0.1 mM PMSF) with bacterially-purified GST or GST-PBX1A HD fusion immobilized for 30 min at 4°C with gentle agitation on glutathione-Sepharose beads (Pharmacia). Beads were washed four times in the presence of NET-N, dried briefly and resuspended in 30 μl 1x loading buffer. Bound proteins were analyzed by SDS-PAGE. The gel was treated with Enhance (Dupont), dried and exposed at -85°C.

3.6 Protease protection assay

^{35}S labelled PBX1A or PBX1A E28R were produced using an SP6 transcription/translation kit (Promega). Reactions were set up in a final volume of 20 μl of 1x reaction buffer (25 mM Tris-HCl (pH 7.9), 50 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM EDTA) with 3 μl of translated proteins. For every sample, the total volume of reticulocyte lysate from the in-vitro translated proteins was 6 μl. After an initial incubation on ice for 30 min, 5 ng of chymotrypsin was added and allowed to digest at 25 ^{0}C for different time points. At every time point, the reactions were stopped with 5x loading buffer and immediately boiled for 3 min. The samples were resolved by 12 % SDS-PAGE. After electrophoresis, the gel was fixed, treated with Amplify (Amersham) for 20 min, dried and exposed to film.

3.7 Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization to 10.5 day mouse embryos was performed as previously described (Folberg et al., 1997).

4. RESULTS AND DISCUSSION

4.1 Mammalian MEIS or PREP proteins direct PBX1A to the nucleus in insect cells

Subcellular localization of HA-tagged PBX1A and MEIS1 (Fig. 1A) was first examined in the *Drosophila* Schneider 2 (S2) cell line. S2 cells were used because they do not express HTH, resulting in the cytoplasmic localization of EXD (Rieckhof et al., 1997). Similar to EXD, PBX1A-HA was strongly cytoplasmic in transfected S2 cells (Fig. 1B), while MEIS1-HA was localized to both the nucleus and cytoplasm (data not shown.) However, when co-expressed, MEIS1 strongly induced the nuclear accumulation of PBX1A-HA (Fig. 1*B*). MEIS2 and MEIS3 also directed efficient nuclear localization of PBX1A-HA (data not shown), as did the MEIS-like protein PREP1 (Fig. 1C). Taken together with other studies on HTH and PREP1 (Abu-Shaar et al., 1999; Berthelsen et al., 1999), we conclude that the entire MEIS protein family is able to reverse the subcellular distribution of PBX1A from cytoplasm to nucleus in S2 cells.

4.2 Residues N-terminal to the HD direct PBX1A to the cytoplasm of S2 cells

To investigate the role of MEIS in the nuclear localization of PBX proteins, we tested two deletions of the PBX1A N-terminus. Residues required for interaction with MEIS map N-terminal to position 89 in PBX (Chang et al., 1997b; Knoepfler et al., 1997). A deletion lacking this domain (Δ1-89-HA) remained strongly localized to the cytoplasm in the absence (Fig. 1B) and presence of MEIS1 (data not shown). By contrast, removal of all residues upstream of the HD (Δ1-232-HA, Fig. 1A) resulted in a

constitutively nuclear protein, even in the absence of MEIS1 (Fig. 1B). Thus, direct association with MEIS1 is unnecessary for nuclear localization of this PBX1A N-terminal deletion mutant. Rather, a region between residues 89 and 232 negatively regulates nuclear accumulation of PBX1A (see below.)

Mammalian Cos-7 cells express both *Meis1* and *Prep1* (data not shown). Unlike S2 cells, full-length PBX1A and Δ1-89 were strongly nuclear in Cos-7. Since the first 89 residues of PBX are required for interaction with the MEIS family, the results demonstrate independence from MEIS/PREP for PBX1A nuclear localization. We conclude that the mechanisms governing the subcellular distribution of PBX proteins may vary between cell types.

4.3 PBX1A contains two cooperative nuclear localization signals in the HD

The above results show that a PBX1A derivative can enter the nucleus after removal of a region required for interaction with MEIS. This suggests that PBX1A does not simply rely on NLS function supplied by MEIS, but has one or more NLS of its own. Analysis of the PBX1A amino acid sequence revealed two potential NLS, both within the HD. The first is located in the N-terminal arm of the HD and represents a good NLS consensus (amino acids 234-239 : RRKRR). The second, spanning residues 285-294 in the third helix, is less conserved (KRIRYKKNI). To test for their function, HA-tagged deletions of PBX1A (Fig. 2A) were assessed in Cos-7 monkey cells (Fig. 2B). A deletion around a presumptive cAMP-dependent phosphorylation site (Δ172-219) did not alter the predominant nuclear localization of the protein (Fig. 2B); however, deletion of a further

35 amino acids removed the first presumptive NLS (Δ172-254) resulting in significant cytoplasmic retention of the derivative protein (Fig. 2B). A larger deletion spanning the entire HD (Δ172-295) removed the second presumptive NLS as well, and showed a more striking effect, with most of the protein remaining in the cytoplasm (Fig. 2B).

Finer mutations of the two NLS were generated. Mutation of the second and third positions of the first NLS from arginine and lysine to leucine and glutamic acid (RK235-236LE), or deletion of the nine amino acids around the second NLS consensus (Δ284-293), resulted in cytoplasmic accumulation (Fig. 2B). The simultaneous presence of both mutations further exacerbated this trend demonstrating cooperativity between the two NLS (Fig. 2B). Overexpression of MEIS1A did not change the subcellular distribution of these mutants (data not shown).

We next examined whether fusion to PBX1A derivatives could direct a heterologous protein, GFP, to the nucleus (Fig. 3A). While GFP was distributed in both the nucleus and cytoplasm, coupling to the PBX HD resulted in complete nuclear localization (Fig. 3B). Mutation of either presumptive NLS resulted in a marked decrease in nuclear accumulation (Fig. 3B) consistent with NLS function. Because the N-terminal arm and third helix contact DNA, it was possible that these regions promoted nuclear retention simply through DNA-binding. We therefore impaired the DNA-binding ability of the PBX HD by converting asparagine 51 to serine N51S, a mutation shown previously to compromise greatly DNA-binding (Lu et al., 1994; Shanmugam et al., 1999; Vershon et al., 1995). We found that nuclear localization by GFP-N51S-HD was comparable to the unmutated fusion protein (Fig. 3B) arguing for true NLS function for both of the sequences examined here.

NLS function has been previously ascribed to the PBX HD, however the NLS were not mapped (Berthelsen et al., 1999). While another study suggested that an NLS may reside at each of the two positions mapped here, no attempt was made to distinguish between passive diffusion into the nucleus versus active NLS-mediated transport (Abu-Shaar et al., 1999). Our study involving GFP fusions clearly demonstrates that the PBX has two cooperative NLS located in the N-terminal arm and helix 3, an observation that has been made for the HD of unrelated proteins as well (Christophe-Hobertus et al., 1999; Hessabi et al., 2000; Parker et al., 2000).

4.4 The PBX1A NLS are sufficient for nuclear localization induced by PREP1

To address the contribution of MEIS or PREP1 to nuclear localization, we examined the subcellular distribution of PBX ΔHD-HA, a PBX1A derivative that lacks the HD and hence the two NLS. Similar to the wildtype protein, PBX ΔHD-HA localized mainly to the cytoplasm in S2 cells (Table 1). Interestingly, the co-expression of MEIS1 but not of PREP1 led to a major nuclear accumulation of the deleted protein (Table 1). This implies the existence of some NLS function in MEIS, as has been shown for its homolog HTH (Abu-Shaar et al., 1999; Jaw et al., 2000). By contrast, PREP1 lacks NLS activity, as also reported by others (Berthelsen et al., 1999). Importantly, this suggests that in the PBX1/PREP1 dimer, it is PBX1A that contributes all NLS function (Fig.1C).

To investigate the role of PBX1A NLS in the PBX1A/MEIS dimer, we examined whether a MEIS ΔHD derivative can direct PBX1A to the nucleus. We found that the N-terminal domain of MEIS1 spanning the PBX interaction domain (residues 1-185) is

capable of directing the majority of PBX1A to the nucleus (Table1). This argues that the NLS function of PBX1A contributes to the nuclear localization of complexes with MEIS as well. However, it should be noted that one of two NLS motifs mapped in HTH is conserved in residues 1 to 185 of MEIS (Jaw et al., 2000). This remaining NLS may have contributed to the nuclear localization of the complex containing PBX.

4.5 Intramolecular interactions block NLS function in PBX1A

Despite the presence of two NLS in the HD, PBX1A is excluded from the nucleus in S2 cells. Interaction with MEIS/PREP1, or removal of the PBX1A N-terminus, reverses this situation. This suggests a model whereby intramolecular contacts between the HD and the PBX1A N-terminus mask the NLS. If correct, such contacts might also impair DNA-binding by PBX. In support of the model, others have shown that deletion of the PBX N-terminus improves DNA-binding by the PBX HD (Calvo et al., 1999; Neuteboom and Murre, 1997). We have confirmed these results. Using EMSA, we assayed for the monomer binding of both wild-type PBX1A and two PBX1A deletion mutants. Full length PBX1A or Δ1-89-HA displayed no detectable monomer binding activity (Fig. 4A, lanes 1, 2 and 3). By contrast, further deletion to 232 markedly increased DNA-binding by the PBX1A HD (lane 4). This is consistent with another study that has more finely mapped a region inhibitory for DNA-binding to 206-232 (Calvo et al., 1999).

If interaction with MEIS exposes the PBX HD, then a non-DNA-binding mutant of MEIS1, MEIS1 N51S, would be predicted to promote DNA-binding by PBX. Confirming our previous observations (Shanmugam et al., 1999), a PBX1A•MEIS1 N51S complex

binds well to an appropriate site (Fig. 4A, compare lanes 2, 5 and 6). The formation of the DNA-bound PBX1A•MEIS1 N51S dimer (lane 6) was dependent on PBX1A DNA-binding activity since PBX1A N51S failed to form a complex with MEIS1 N51S (lane 7). Thus, interaction with a DNA-binding-impaired mutant of MEIS1 greatly increases DNA-binding activity by PBX1A, an effect necessarily mediated by a change in conformation.

To examine physical association between the PBX HD and region 1-232, we performed GST-pull-down experiments. Whereas GST alone brought down only trace quantities of region 1-232 (Fig. 4B, lane 2), a GST-HD fusion protein displayed robust interaction with this same domain (Fig. 4B, lane 3). Specificity was further demonstrated by the lack of interaction of GST-HD with fire-fly luciferase (Fig. 4B) or with a smaller PBX1 domain consisting of the first 96 amino acids only (data not shown). These results confirm the direct physical association between the PBX N-terminus and HD implied by earlier experiments. Together, these results support a model whereby interaction between the PBX N-terminus and HD blocks both DNA-binding and nuclear localization. Both functions would be activated by exposure of the HD induced by interaction of MEIS/PREP with the PBX N-terminus.

4.6 A mutation in the PBX1A HD results in constitutive nuclear localization

The PBX HD comprises 63 residues with an insertion of three residues typical of the TALE class HD (Bürglin, 1997). A mutation in the second α-helix that converts glutamic acid to arginine (E28R, corresponding to position 263 in full length PBX1A)

enhances the monomeric binding of a truncated PBX1A (Calvo et al., 1999). We introduced the E28R mutation into full-length PBX1A and found that this protein likewise displayed increased DNA-binding as a monomer (Fig. 4A, lane 8). As previously suggested, mutation of position 28 may disrupt intramolecular contact between the PBX N-terminus and HD (Calvo et al., 1999). An untested prediction of this hypothesis is that this same mutation should also unmask the NLS in the PBX HD, resulting in constitutive nuclear localization of PBX (Calvo et al., 1999). We found this to be the case: full-length PBX1A bearing the E28R mutation was constitutively nuclear in S2 cells in the absence of MEIS/PREP (Fig. 5A).

If the E28R mutation does relax interaction between the PBX N-terminus and HD, as suggested by the above results, then the mutant protein should display an altered accessibility to proteases. As seen in figure 5B, the E28R mutation significantly increased the rate of digestion by chymotrypsin in two independent experiments. While the E28R mutant was almost fully digested in only 2 minutes, significant amounts of the wild type protein remained at this time point. In addition, the relative proportion of digestion products was altered, since prominent bands observed in the E28R digest are barely visible with wild type. This demonstrates an altered configuration induced by the E28R mutation consistent with loss of interaction between the PBX N-terminus and HD.

It should be noted that the ability of the E28R mutant to bind DNA argues against a significant alteration in the configuration of the HD itself. Moreover, position 28 has not been directly implicated in maintaining the integrity of the PBX HD (Passner et al., 1999; Piper et al., 1999). It has been suggested that a cluster of glutamic acid residues in helix 2 of EXD, including E28, contributes to electrostatic repulsion of DNA. The replacement

of a negative charge with a positive one in the E28R mutant may therefore improve DNA binding by decreasing electrostatic repulsion (Passner et al., 1999). However, our results with the E28R mutant showing increased susceptibility to protease digestion and constitutive nuclear localization argue against this possibility. Together, our findings strongly support a model in which binding by MEIS/PREP to the PBX N-terminus induces a conformational change that exposes the PBX HD, thereby increasing DNA-binding activity and unmasking the two NLS (Fig. 6). Conformational change leading to NLS exposure has been shown for other proteins upon ligand binding (Ylikomi et al., 1992 and references therein) or phosphorylation (Robbins et al., 1991).

4.7 Nuclear export is another determinant of PBX1A subcellular distribution in S2 cells

LMB is an antibiotic that specifically inhibits CRM1-mediated nuclear export (Fornerod et al., 1997a; Nishi et al., 1994). As demonstrated by others (Abu-Shaar et al., 1999; Berthelsen et al., 1999), LMB induced substantial nuclear accumulation of PBX1A in S2 cells in the absence of MEIS (data not shown.) Nuclear localization of PBX1A was incomplete upon LMB treatment, with virtually no cells showing complete nuclear localization. Moreover, raising the concentration of LMB from 100 nM to 250 nM did not change the fraction of PBX1A remaining in the cytoplasm. A similar observation has been made for the response of EXD to LMB (Abu-Shaar et al., 1999). These results argue for another mechanism, in addition to nuclear export, in the control of the subcellular distribution of PBX. This is consistent with our model whereby NLS function is blocked

by the PBX N-terminus. Non-exclusively, a cytoplasmic retention factor could restrict PBX to this compartment.

We (this study) and others (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Calvo et al., 1999; Neuteboom and Murre, 1997) have shown that a region N-terminal to the PBX HD inhibits both DNA-binding and nuclear localization. Whereas region 206-231 has been suggested to inhibit DNA-binding by contacting the HD (Calvo et al., 1999), the overlapping region 174-214 (179-219 in EXD) has been proposed to harbor an NES (Abu-Shaar et al., 1999). We confirmed that an internal deletion of this domain (Δ172-219) does indeed result in constitutive nuclear localization in S2 cells (Fig. 1). Given the results presented above, it seems likely that this small region excludes PBX from the nucleus for two reasons: it harbors an NES, and it blocks NLS function. In addition to exposing the NLS, interaction with MEIS/PREP may also mask the NES, further swinging the balance in favor of nuclear import over export.

Our studies imply a major conformational change in PBX1A upon interaction with MEIS1. Additional intermolecular contacts to DNA and the HOX YPWM motif have also been shown to alter the configuration in and around the PBX1 HD (Jabet et al., 1999; Sprules et al., 2000). Together with our results, these observations suggest that the PBX HD is the focus of a series of conformational changes that are necessary to fulfill multiple roles.

4.8 *Meis1* expression correlates with PBX1 nuclear localization in the mouse embryo

If MEIS1 is required for the nuclear localization of PBX in mammals, then nuclear PBX should be restricted to sites of *Meis1* expression. PBX1 is nuclear only in the proximal limb bud, cells of the distal limb bud retaining PBX1 in the cytoplasm (Gonzalez-Crespo et al , 1998). Whole-mount *in situ* hybridization with a probe spanning the *Meis1* cDNA reveals that *Meis1* transcripts are indeed found in the proximal limb bud where PBX1 is nuclear, but not distally where PBX1 is cytoplasmic (Fig. 7). While we have only assayed for *Meis1* transcripts and not MEIS1 protein, the correlation between the domains of *Meis1* expression and nuclear localization of PBX1 is striking. This was not due to differential access to the probe since a control experiment using a probe from the murine *Hoxd4* gene showed no proximo-distal restriction in expression pattern, whereas a probe for the murine *Msx1* gene (Hill et al., 1989) showed appropriate distally restricted expression in the limb bud (data not shown). *Meis2* transcripts are also proximally restricted in the mouse limb bud (Cecconi et al., 1997; Oulad-Abdelghani et al., 1997), similar to what we report here for *Meis1*. HTH is likewise restricted to the proximal leg primordia in flies where it is required for nuclear localization of EXD (Pai et al., 1998; Rieckhof et al., 1997). Together with the demonstration that MEIS1 (this study), and PREP1 (this study and Berthelsen et al., 1999) direct PBX1 to the nucleus of cultured cells, it appears that the entire MEIS/PREP/HTH family plays a conserved role in controlling the subcellular distribution of PBX/EXD.

5. ACKNOWLEDGMENTS

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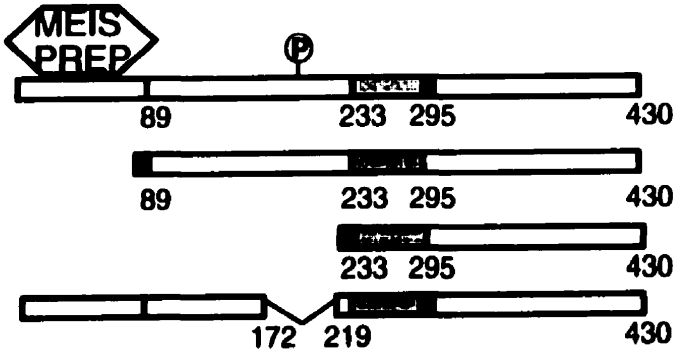
Figure 1. Interaction with MEIS1, or loss of PBX1A region 1-232, is sufficient to direct PBX1A to the nucleus.

(A) Schematic representation of full-length PBX1A tagged with the HA epitope (PBX1A-HA), N-terminally truncated mutants Δ1-89-HA and Δ1-232-HA and the internal deletion mutant Δ172-219-HA. The HD is indicated by a gray box extending from amino acids 233 to 295. The black box represents the position of the HA tag. The region extending from amino acids 1 to 89 encompasses the domain for interaction with both MEIS and PREP1.

(B) *Drosophila* Schneider (S2) cells, that do not express HTH, were transiently transfected with insect expression vectors for PBX1A-HA (with and without a MEIS1 expression vector), Δ1-89-HA, Δ1-232-HA and Δ172-219-HA and subsequently analyzed by immunohistochemistry using anti-HA (HA-11) monoclonal antibody. Two representative photomicrographs are presented for each construct tested. Cells were classified into 3 categories: "N" denotes cells presenting staining only in the nucleus. "N>C" denotes cells with nuclear staining stronger than cytoplasmic staining and "N≤C" represents cells in which nuclear staining is equal to, or less than, that of cytoplasm. The numbers presented here are percentages of cells falling into each category with a total of at least 250 transfected cells scored. Within brightly fluorescing nuclei, the nucleolus is seen as a small unstained circle, as also noted in Cos-7 cells (Fig. 3).

(C) The MEIS-related protein PREP1 can direct the nuclear localization of PBX1A Images are confocal photomicrographs with a final magnification of 1100x in (B) and 1600x in (C).

A



B

			N	N>C	N<C
PBX1A-HA	S2		-	7	92
PBX1A-HA + MEIS1	S2		66	22	12
Δ1-89-HA	S2		7	33	60
Δ1-232-HA	S2		82	6	12
Δ172-219-HA	S2		70	12	18

C

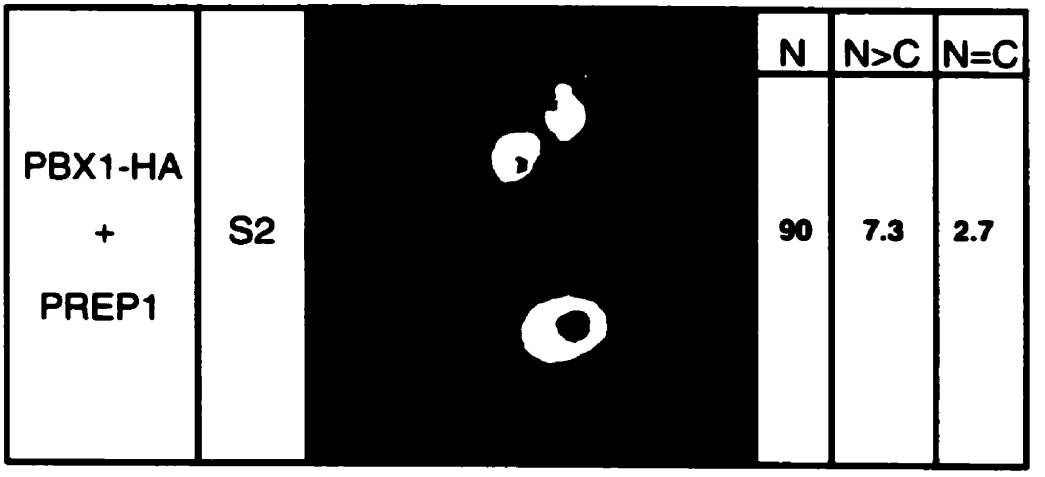


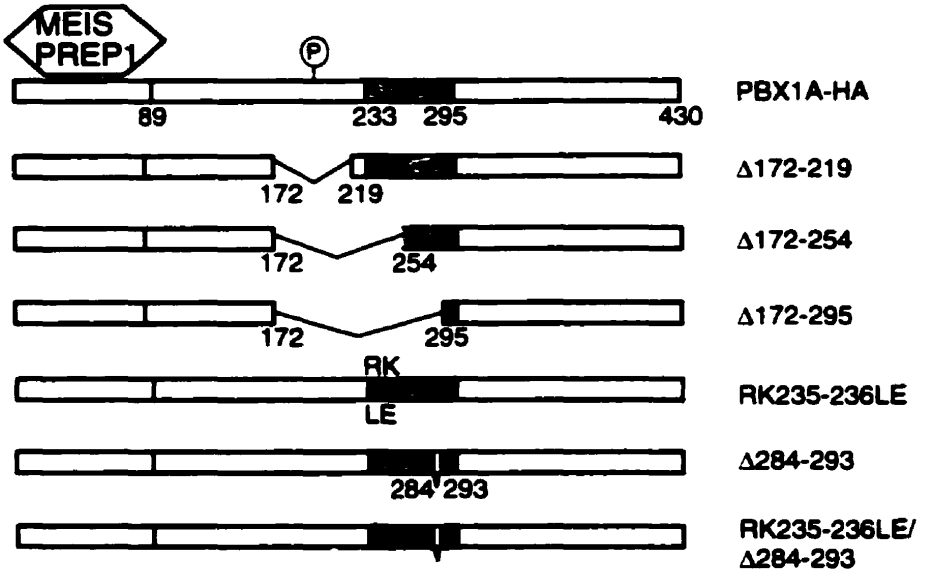
Figure 2. PBX1A harbors two nuclear localization signals in the HD.

(A) Schematic representation of full-length PBX1A-HA along with internal deletions and point mutations around its HD (gray box). In all cases, MEIS and PREP1 are expected to interact with the mutant derivatives since region 1-89 is intact. The position of the HA tag (black box) is constant.

(B) Immunohistochemistry using the anti-HA monoclonal antibody (HA-11) showing cellular compartmentalization of PBX1A derivatives in Cos-7 cells. N, N>C and N≤C as for Figure 1B, "C" represents cells with no detected staining in the nucleus. Images are confocal photomicrographs with a final magnification of 550x.

(C) Subcellular distribution of PBX1A derivative Δ1-89-HA in Cos-7 cells.

A



B

			N	N>C	N<C	C
PBX1A-HA	Cos-7		95	5	-	-
Δ172-219	Cos-7		95	5	-	-
Δ172-254	Cos-7		-	29	71	-
Δ172-295	Cos-7		-	25	53	22
RK235-236LE	Cos-7		7	21	72	-
Δ284-293	Cos-7		-	14	86	-
RK235-236LE/ Δ284-293	Cos-7		-	14	71	15

C

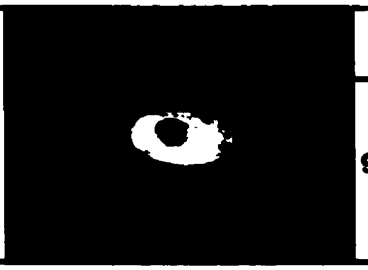
			N	N>C	N≤C
Δ1-89-HA	Cos-7		95.5	4.5	-

Figure 3. Both NLS of PBX1A are required to carry GFP to the nucleus.

(A) Schematic representation of wild-type GFP and GFP-fusion proteins. In GFP-HD, PBX1A region 219-295 is fused C-terminal to GFP. RK235-236LE is identical to GFP-HD except for point mutations in the second and third residues of the first NLS. In Δ286-295, the last nine residues of the HD encompassing the second NLS are deleted. In GFP-N51S-HD, asparagine residue 51 of the HD is converted to serine.

(B) Detection of GFP fluorescence under UV light using confocal photomicrography with a final magnification of 360x. Cos-7 cells were transiently transfected with GFP or GFP-fusion proteins and the cellular compartmentalization of the proteins was denoted as N, N>C and N≤C as for Figure 1B. "C" represents cells with no detectable staining in the nucleus.

Figure 4. Intramolecular interactions in PBX1A.

(A) EMSA analysis examining monomer binding of *in-vitro* translated wild-type PBX1A (lane 2), PBX1A N-terminal deletion derivatives Δ1-89 (lane 3) and Δ1-232 (lane 4), PBX E28R (lane 8), PBX1A in the presence of MEIS1 (lane 5), PBX1A in the presence of MEIS1 N51S (lane 6) and PBX1A N51S in the presence of MEIS1 N51S (lane 7) on a ^{32}P-labeled "G6" probe (P): TGATTGAT (underlined) contains binding sites for PBX (TGATTG) and HOX proteins (TGATGG) but no consensus binding site for MEIS protein (TGACAG Chang et al., 1997b). "Mock" (lane 1) refers to a translation reaction to which no template was added. A non-specific band seen in all lanes is likewise labeled "Mock". This band is intense since no poly dI•dC was added to the reactions in order to maximize binding by monomeric PBX derivatives. DNA-bound PBX E28R is seen in lane 8 as a strong band migrating just faster than the mock band. The asterisk notes the position of a possible homodimer of PBX E28R.

(B) GST pull-down experiments. A purified fusion protein consisting of GST fused to the PBX1A HD (residues 233 to 295) was incubated with either labeled PBX1A 1-232 (lanes 2 and 3) or labeled luciferase (lanes 5 and 6). The input lanes 1 and 4 represent 10% of the total amount of the labeled proteins used in each binding reaction. Equivalent amounts of labeled protein were used in lanes 2, 3, 5 and 6.

(C) Coomassie staining of the gel presented in (B) as a control for equal protein loading.

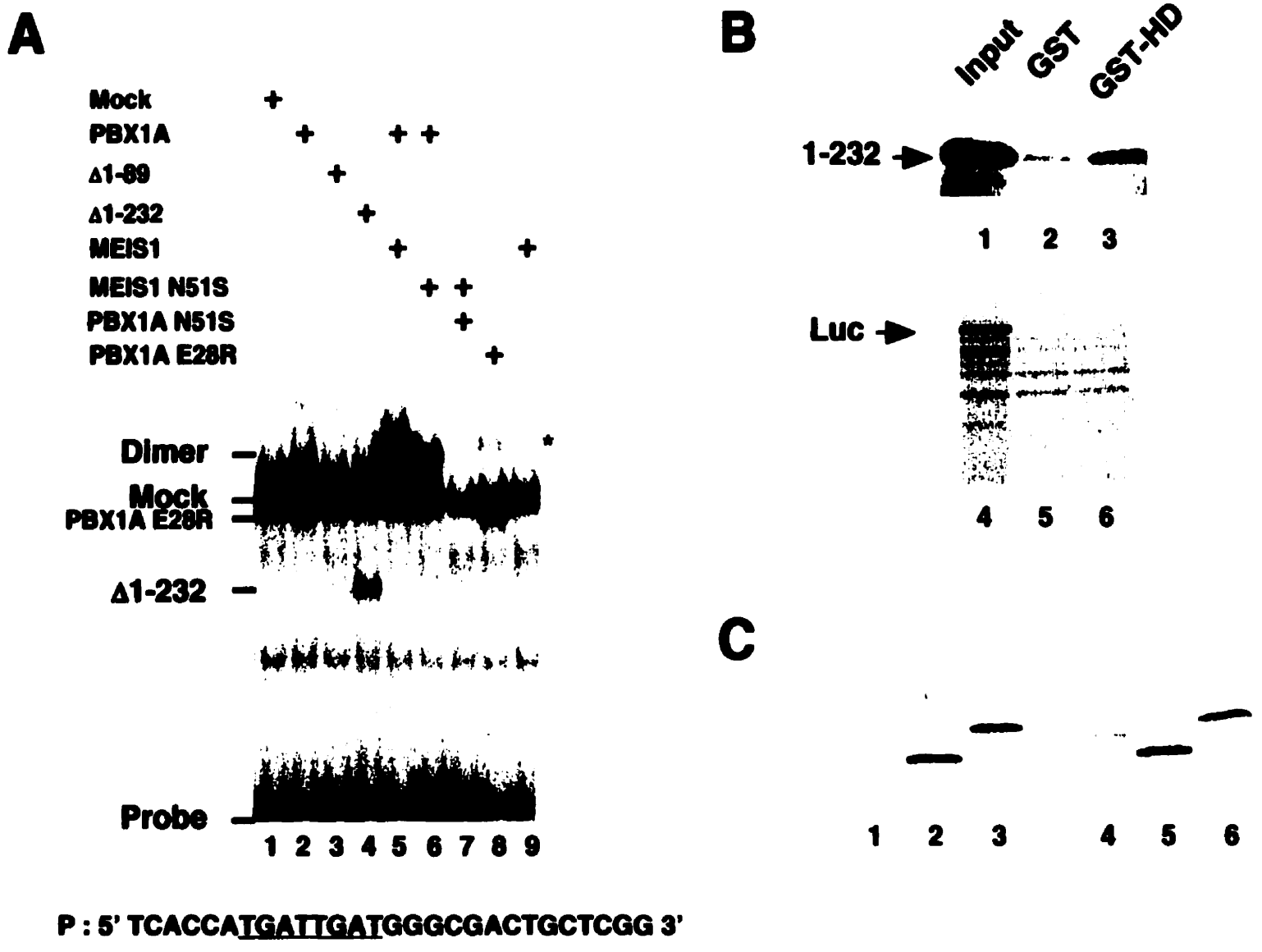
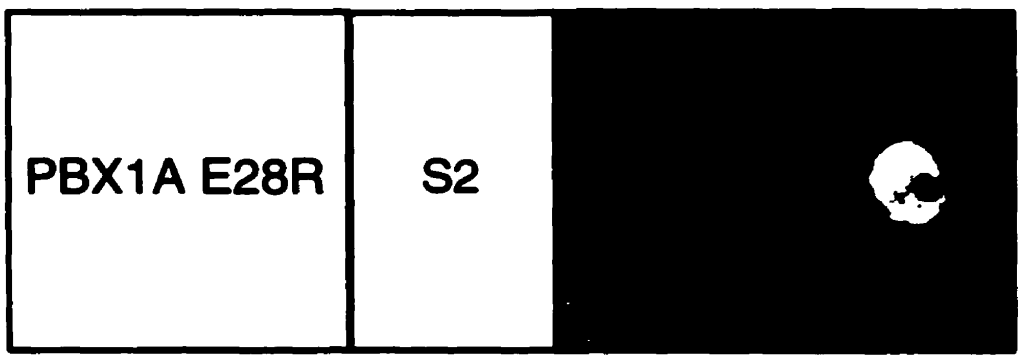

A
Mock
PBX1A
Δ1-89
Δ1-232
MEIS1
MEIS1 N51S
PBX1A N51S
PBX1A E28R
Dimer
Mock
PBX1A E28R
Δ1-232
Probe
1 2 3 4 5 6 7 8 9
B
Input
GST
GST-HD
1-232
1 2 3
Luc
4 5 6
C
1 2 3 4 5 6
P : 5' TCACCATGATTGATGGGCGACTGCTCGG 3'

Figure 5. An E28R mutation in PBX1A HD induces a conformational change in the protein and results in its constitutive nuclear localization.

(A) Immunohistochemistry using anti-PBX1A polyclonal antibodies showing nuclear staining of PBX1A E28R. The image is a confocal photomicrograph with a final magnification of 1100x.

(B) PBX1A and PBX1A E28R were labelled with ^{35}S during *in vitro* translation and incubated with 5 ng of chymotrypsin for the indicated time points at 25 ^{0}C. The cleavage products were resolved by 12 % SDS-PAGE. Dots indicate protease-induced bands that are much more prominent in digests of PBX1A E28R than wild type.

A



B

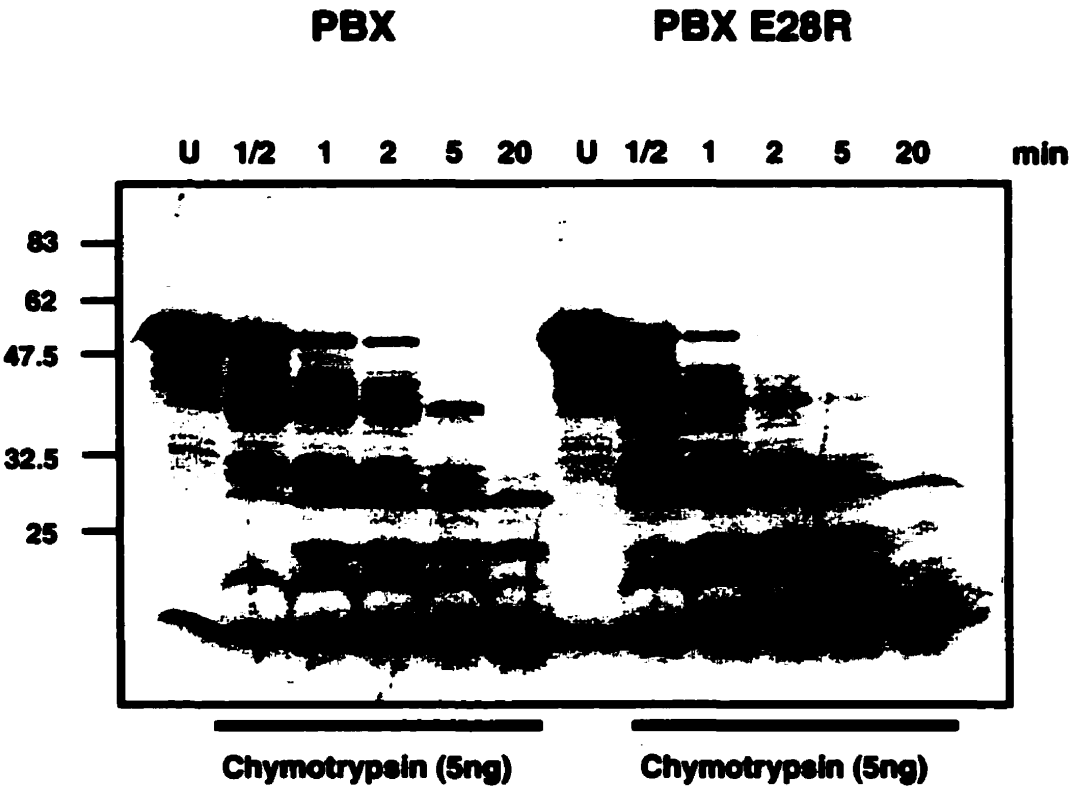

PBX
PBX E28R
U 1/2 1 2 5 20 U 1/2 1 2 5 20 min
83
62
47.5
32.5
25
Chymotrypsin (5ng)
Chymotrypsin (5ng)

Figure 6. Model for the regulation of PBX1A nuclear localization by HTH homologs.

The PBX1A HD, represented by the three cylinders, contains two NLS (black circles) in the N-terminal arm and at the C-terminus of helix 3. In the absence of an HTH homolog such as MEIS1 or PREP1, the PBX1A NLS are masked by the PBX N-terminus (rectangle), resulting in cytoplasmic localization. Binding of MEIS or PREP1 to region 1-89 of PBX1A (gray box) leads to a conformational change in PBX1A, and perhaps to the displacement of a cytoplasmic retention factor, that exposes the NLS. This results in PBX1A nuclear localization. In addition, interaction with MEIS or PREP1 blocks one or more nuclear export signals located between residues 1 to 89 and/or 172-219 of PBX. Access to the nuclear import machinery combined with an inhibition of nuclear export results in efficient nuclear accumulation.

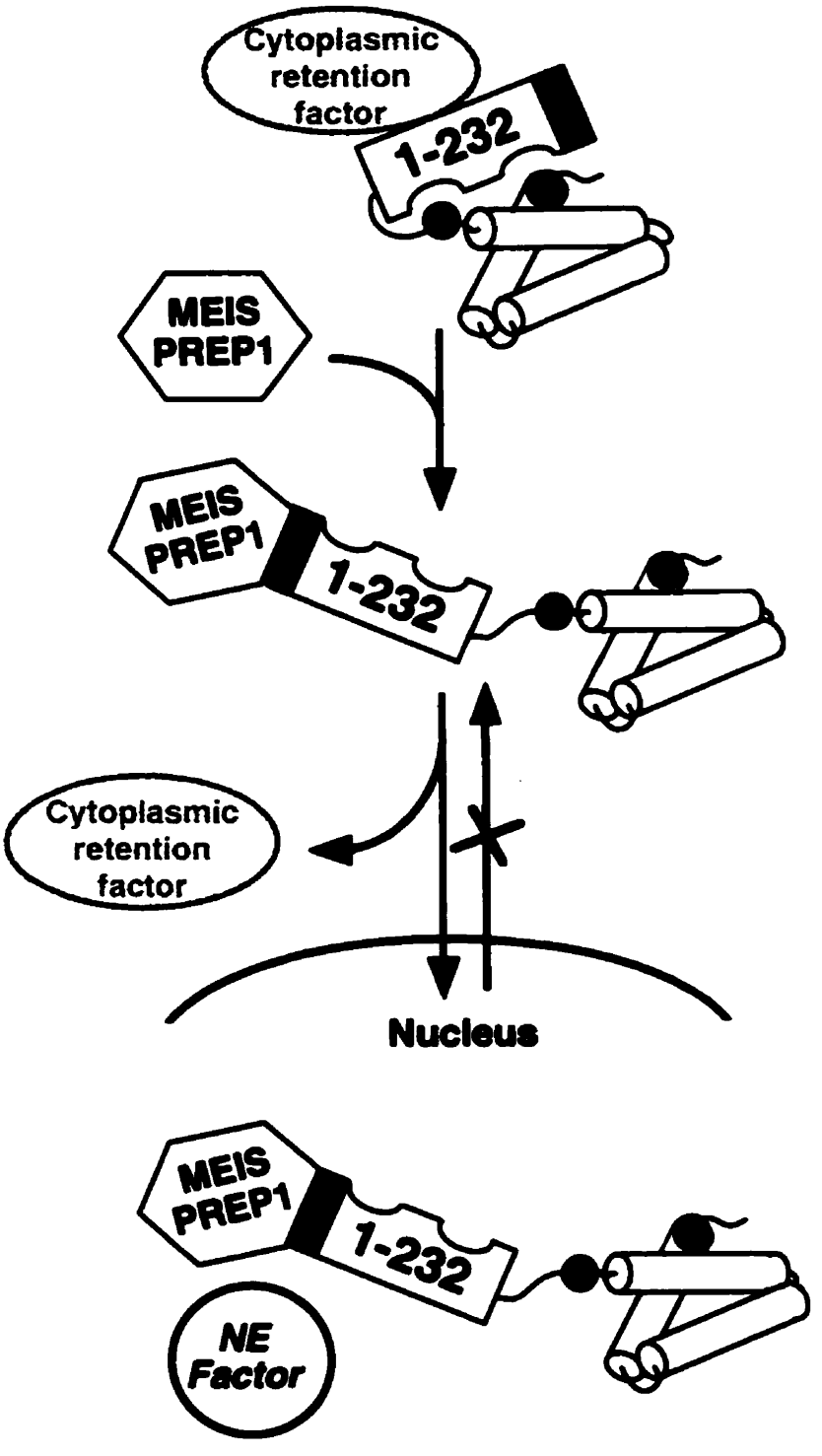

Cytoplasmic retention factor
1-232
MEIS PREP1
MEIS PREP1
1-232
Cytoplasmic retention factor
Nucleus
MEIS PREP1
1-232
NE Factor

Figure 7. *Meis1* expression is restricted to sites of PBX1 nuclear localization.

A 10.5 day mouse embryo was used in whole-mount *in situ* hybridization with an anti-sense probe spanning the *Meis1a* coding region. A dorsal view over the forelimb buds shows that the *Meis1* signal is strong over the proximal limb bud, where PBX1 has been shown to be nuclear (Gonzalez-Crespo et al., 1998). By contrast, no signal is detected in the distal limb bud where PBX1 is cytoplasmic.

fig

fig

TABLE 1

Subcellular distribution in S2 cells of PBX1A derivatives containing or lacking the HD in the presence of MEIS, PREP1, and a MEIS derivative lacking the HD.

	N	N>C	N=C
PBX1 ΔHD-HA	-	7	93
PBX1 ΔHD-HA + PREP1	-	13	87
PBX1 ΔHD-HA + MEIS1	64	25	11
PBX1-HA + MEIS1(1-185)	77	19	4

CONNECTING TEXT

The nuclear localization of PBX is essential for its function. Only in the presence of MEIS/PREP1 protein is PBX in the nucleus. In the last chapter, we investigated the mechanisms of regulation of PBX nuclear availability by MEIS/PREP1 and showed that MEIS/PREP1 induces a conformational change in PBX that is required to expose its otherwise masked NLS. In the nucleus, PBX functions with MEIS/PREP1 or with HOX proteins to regulate the transcription of downstream targets. In the next chapter, we address the mechanisms of transcriptional regulation by the HOX•PBX complex. We show that the HOX•PBX complex represses or activates transcription by differential recruitment of transcriptional coregulators. In a HOX•PBX heterodimer, PBX recruits a corepressor complex composed of N-coR/SMRT•mSIN3B•HDAC to mediate transcriptional repression, while HOX recruits the coactivator CBP to activate transcription. Cell aggregation, PKA signaling or inhibition of cellular HDACs switch the HOX•PBX complex from transcriptional repressor to activator.

CHAPTER III

Cell Signaling Switches HOX•PBX Complexes from Repressors to Activators of Transcription Mediated by Histone Deacetylases And Acetyltransferases

Maya Saleh, Isabel Rambaldi
Xiang-Jiao Yang and Mark S. Featherstone

Molecular and Cellular Biology 20 (22), 8623-8633 (2000)

1. ABSTRACT

The *Hoxb1* autoregulatory element (ARE) comprises three HOX•PBX binding sites. Despite the presence of HOXB1 and PBX1, this enhancer fails to activate reporter gene expression in RA-treated P19 cell monolayers. Activation requires cell aggregation in addition to RA. This suggests that HOX•PBX complexes may repress transcription under some conditions. Consistent with this, multimerized HOX•PBX binding sites repress reporter gene expression in HEK293 cells. We provide a mechanistic basis for repressor function by demonstrating that a corepressor complex including histone deacetylases (HDAC) 1 and 3, mSin3B and N-CoR/SMRT interacts with PBX1. We map a site of interaction with HDAC1 to the PBX1 N-terminus, and show that the PBX partner is required for repression by the HOX•PBX complex. Treatment with the deacetylase inhibitor trichostatin A (TSA) not only relieves repression, but converts the HOX•PBX complex to a net activator of transcription. We show that this activation function is mediated by the recruitment of the coactivator CREB-binding protein (CBP) by the HOX partner. Interestingly, HOX•PBX complexes are switched from transcriptional repressors to activators in response to protein kinase A (PKA) signaling or cell aggregation. Together, our results suggest a model whereby the HOX•PBX complex can act as a repressor or activator of transcription *via* association with corepressors and coactivators. The model implies that cell signaling is a direct determinant of HOX•PBX function in the patterning of the animal embryo.

2. INTRODUCTION

HOX proteins are sequence-specific DNA-binding transcription factors that play a crucial role in the specification of antero-posterior identity in the animal embryo (Favier and Dollé, 1997; McGinnis and Krumlauf, 1992). Conservation within the DNA-binding homeodomains results in different HOX proteins recognizing similar regulatory elements with only modest preferences (reviewed in Graba et al., 1997). High-affinity DNA-binding is achieved when HOX proteins are heterodimerized with partners of the PBC family (mammalian PBX, *Drosophila* Extradenticle (EXD) and Caenorhabditis elegans CEH-20) (Monica et al., 1991). Mammalian MEIS1 has been shown to independently dimerize with HOX proteins and with PBX (Chang et al., 1997b; Moskow et al., 1995; Shen et al., 1997a). Recently, trimeric complexes encompassing all three homeoproteins, HOX•PBX•MEIS, have also been characterized (Shanmugam et al., 1999; Shen et al., 1999). The MEIS-related protein PREP1, also known as PKNOX1, can additionally form a dimer with PBX as well as a trimeric complex with HOX and PBX partners (Berthelsen et al., 1998a; Berthelsen et al., 1998b; Chen et al., 1997b; Knoepfler et al., 1997). While the majority of HOX monomers recognize a DNA core motif of TAAT (Gehring et al., 1994b), HOX•PBX, HOX•MEIS and PBX•MEIS heterodimers recognize larger motifs resulting in higher affinity and specificity of DNA-binding by these homeoproteins (Mann and Affolter, 1998).

A conserved motif with the consensus YPWM is found N-terminal to the homeodomain of HOX proteins from paralogous groups 1-8. The YPWM motif contacts the PBX homeodomain and is strictly required for cooperative DNA-binding by PBX and

HOX partners (Mann and Affolter, 1998; Mann and Chan, 1996). A conserved W in HOX proteins from groups 9 and 10 performs a similar function (Chang et al., 1995).

The downstream targets of mammalian HOX proteins have been poorly characterized. The best characterized targets are some *Hox* genes known to be positively autoregulated by their own products or cross-regulated by the products of other *Hox* genes (Gould et al., 1997; Pöpperl et al., 1995; Pöpperl and Featherstone, 1992). In these instances, HOX•PBX complexes act as activators of transcription. For example, the *Hoxb1* ARE contains three binding sites for HOX•PBX complexes. These sites are required to direct expression of a *Hoxb1* transgene in rhombomere r4 of the developing hindbrain (Pöpperl et al., 1995).

Genetic and molecular studies have provided evidence supporting a negative regulatory role for HOX proteins (Li et al., 1999). In the case of *decapentaplegic (dpp)* regulation in *Drosophila*, repression by HOX proteins dominates over activation (Capovilla, 1998). This implies active transcriptional repression by HOX proteins (Capovilla, 1998; González-Reyes et al., 1992; Lufkin et al., 1991). In addition, *in vitro* mapping studies have characterized repression domains in different HOX proteins as well as in the PBX partner (Chariot A, 1996; Lu and Kamps, 1996a; Schnabel, 1996). Therefore, HOX proteins may be activators or repressors in a context-dependent manner.

By analogy to nuclear receptors, HOX•PBX complexes are likely to achieve transcriptional repression or activation through differential association with coactivators and corepressors (Struhl, 1998). One class of coregulators are the histone acetyltransferases (HATs) and the deacetylases (HDACs) which modify chromatin as well as non-histone proteins. The HATs include GCN5, PCAF, CBP/p300, the steroid

receptor coactivator (SRC) class, and the MYST family (Sterner and Berger, 2000). On the other hand, the known HDACs include HDAC1 through 8, with class I HDACs consisting of HDAC1, HDAC2, HDAC3 and HDAC8 (homologues of the yeast RPD3 protein) and class II HDACs including HDAC4, HDAC5, HDAC6 and HDAC7 (homologues of the yeast HDA1 protein) (for review see Kouzarides, 1999). HDAC1 and HDAC2 form the catalytic subunits of two characterized multi-protein complexes, the mSIN3A and Mi2 complexes (Knoepfler and Eisenman, 1999). Additionally, HDAC3 has been shown to interact with the corepressor SMRT (Guenther et al., 2000). Recent genetic evidence in *C-elegans* shows EGL-27, a homologue of MTA1 (a component of the Mi2-HDAC1 complex), in the same pathway as MAB-5 (Xue et al., 1998; Zhang et al., 1998a), further implying that HOX proteins may interact with HDACs and other histone modifying enzymes to accomplish their developmental program.

In this report, we present evidence for an interaction between HOX•PBX complexes and histone modifying enzymes and show that the activity of the HOX•PBX heterodimer is determined by a regulated balance between a corepressor complex consisting of class I HDACs, mSIN3B and N-CoR/SMRT and a coactivator complex containing CBP. We show, moreover, that activation of the PKA signaling pathway significantly potentiates the CBP-mediated transactivation by HOX•PBX complexes. We propose a model in which PKA acts as a signaling switch that converts HOX•PBX complexes from transcriptional repressors to activators.

3. MATERIALS AND METHODS

3.1 Cell culture and transfections

Mouse embryonic carcinoma P19 cell-line and human embryonic kidney HEK 293 cell-lines were cultured in α-minimal essential medium supplemented with 10 % fetal calf serum. Transient transfections were performed using the calcium phosphate precipitation method as described in (Rambaldi et al., 1994). A *lacZ* reporter driven by the cytomegalovirus (CMV) enhancer was used to control for transfection efficiency in some experiments. Because the activity of the CMV enhancer appeared to change in response to PKA, a *lacZ* reporter driven by the Rous sarcoma virus (RSV) long terminal repeat (LTR) was used in transfections involving PKA. For stable transfections of P19, the cells were seeded at a density of 10^5 cells/10cm plate and transfected with a total of 15 μg DNA consisting of 9 μg of the transgene of interest (p1230 or b1-ARE-lacZ), 1 μg of PGK-puromycin and 5 μg pCAB-B17 as the carrier DNA (McBurney et al., 1994; McBurney et al., 1998). 40 h post-transfection, cells were selected with 2 μg/ml puromycin for at least 10 days. Cells were kept in monolayer or aggregated in bacterial petri dishes for 24 h in the presence or absence of treatment, then re-attached in tissue-culture plates O/N. The treatment consisted of either RA ($3x10^{-7}$M) or TSA (concentrations ranging from 20nM to 2μM) or a combination of both RA+TSA. Significant cell death sometimes occurred in response to TSA, however this was variable and dependent on drug concentration and cell context. HEK293 cells were more sensitive than P19 to TSA-induced cell death. Cells were treated with the estrogen antagonist α-hydroxytamoxifen (TOT) overnight at 10^{-7} M.

3.2 Antibodies

Rabbit polyclonal antibodies raised against PBX1, mSIN3A or mSIN3B were purchased from Santa Cruz. Rabbit polyclonal antibodies against human HDAC1 and HDAC3 were from Upstate Biotechnology. Rabbit polyclonal antibodies against HOXB1 were generously supplied by C. Largman. Mouse monoclonal antibodies against the GAL4 DNA-binding domain (DBD) (RK5C1), the hemagglutinin epitope (HA-11), and the flag epitope (M2) were purchased from Santa Cruz, Babco and Sigma, respectively. Mouse monoclonal antibodies were recognized with horseradish peroxidase (HRP)-conjugated goat anti-mouse (κ light chain) secondary antibodies from PharMingen and rabbit polyclonal antibodies were recognized by HRP-conjugated protein A sepharose (Amersham).

3.3 Plasmids

p1230 is a *lacZ* reporter under the control of the minimal promoter of the β globin gene. b1-ARE-lacZ consists of the ARE of the *Hoxb1* gene (Pöpperl et al., 1995) cloned by PCR amplification into the *Hin*dIII-*Xho*I sites of p1230. pML, pML(5xHOX•PBX), pML5xHOX and pML5xUAS are luciferase reporters containing the adenovirus major late promoter alone, driven by 5X HOX•PBX binding sites (TGATTGAT), 5X HOX binding sites (TAAT), or 5X GAL4 binding sites, respectively (Phelan et al., 1995; Rambaldi et al., 1994; Shanmugam et al., 1999). Expression plasmids for HOXA1, HOXD4, PBX1A and PBX1A deletion mutants have been previously described (Phelan and Featherstone, 1997; Shanmugam et al., 1999). The HOXB1 expression vector is driven by the beta-actin promoter. 89-172-HA was constructed by PCR amplification of

region 89-172 followed by cloning of the product in frame with 3X of the HA epitope, in the plasmid pRC/CMV (Invitrogen). Flag-HDAC1, flag-HDAC3 and E1A are described elsewhere (Yang et al., 1996a; Yang et al., 1997; Yee et al., 1983) and were generously provided by Albert Lai (McGill university). Flag-HDAC4 and flag-PCAF are described elsewhere (Wang et al., 1999; Yang et al., 1996b). Flag-N-CoR, flag-SMRT, HA-CBP and the CBP domains were generously provided by Vincent Giguère and André Tremblay (McGill University, Université de Montréal). GAL4-HOXD4N fuses the first 141 residues of HOXD4 to the GAL4 DBD and was described previously (Rambaldi et al., 1994). HOXD4 residues 139 to 250 were fused to the GAL4 DBD to generate GAL4-HOXD4C. An expression vector for the human estrogen receptor alpha driven by the CMV enhancer was generously provided by Vincent Giguère (McGill University).

3.4 β-Galactosidase and luciferase assays

Luciferase assays and liquid β-galactosidase assays were performed as described previously (Phelan et al., 1995). β-galactosidase plate assays were performed after fixation of the cells with a solution of 2 % formaldehyde/ 0.2 % glutarylaldehyde in PBS for 5 min at 4 degrees C. The cells were washed with PBS for three times and then stained at 37 degrees C with a solution composed of 5mM ferrocyanide, 5mM ferricyanide, 1mg/ml X-gal and 2mM $MgCl_2$ in PBS.

3.5 Immunoprecipitation assays

40 h post-transfection, the cells were harvested and lysed on ice for 30 min with 500 µl of a low stringency buffer containing 150 mM KCl. Whole cell extracts were pre-

cleared with protein A or protein G sepharose (depending on the source of the primary antibody used) for 30 min. Pre-cleared lysates were incubated with 0.5-2 μg of primary antibody for 2 h followed by the addition of 20 μl 50 % slurry of protein A or protein G sepharose for 2-18 h. Precipitates were washed 6 times with the lysis buffer and eluted by boiling in 2X sample buffer for 15 min. Eluted proteins were resolved by SDS-PAGE electrophoresis and analyzed following western blotting to polyvinylidene difluoride membranes (Millipore). Secondary antibodies used in western were HRP-conjugated and were detected by enhanced chemiluminescence (NEN Life Science). To immunoprecipitate flag-epitope-tagged proteins, similar protocol was used except that M2 beads (Sigma) were used instead of protein G sepharose and flag peptides (Sigma) were used to elute the precipitated proteins prior to boiling.

4. RESULTS

4.1 TSA relieves the transcriptional repression of HOX•PBX-responsive enhancers

The induction of *Hoxb1* upon RA treatment of mouse embryos is mediated directly by a 3' RA response element (RARE) (Marshall et al., 1994), and indirectly by an ARE (Pöpperl et al., 1995). The *Hoxb1* ARE consists of three cooperative binding sites for HOX•PBX heterodimers (Fig. 1A, top panel). Two paralog group 1 HOX proteins, HOXB1 (Di Rocco et al., 1997) and HOXA1 (M. Phelan and M.S.F., unpublished observations), can activate transcription through the *Hoxb1* ARE. Both gain- and loss-of-function experiments show that HOXA1 and HOXB1 regulate *Hoxb1* expression in the embryonic hindbrain (Barrow et al., 2000; Pöpperl et al., 1995; Studer et al., 1998; Zhang et al., 1994). These effects are very likely to be mediated by the *Hoxb1* ARE as has been demonstrated in one case (Pöpperl et al., 1995). In addition to HOXB1 and PBX, coexpression of PREP1 stimulates reporter gene expression through the *Hoxb1* ARE in transfected cells (Berthelsen et al., 1998a). Together, these results suggest that the presence of first group HOX proteins, PBX, and members of the MEIS/PREP family would be sufficient to activate transcription through the *Hoxb1* ARE.

P19 embryonal carcinoma (EC) cells differentiate along the neural pathway when aggregated in the presence of RA (Jones-Villeneuve et al., 1983; McBurney and Rogers, 1982). While RA-treated P19 cell monolayers fail to form neurons and glia, the products of the *Hoxb1*, *Hoxa1*, *Pbx*, *Meis* and *Prep* genes are induced (Ferretti et al., 2000; Knoepfler and Kamps, 1997; LaRosa and Gudas, 1988; Oulad-Abdelghani et al., 1997).

We therefore expected that a stably integrated transgene carrying the *Hoxb1* ARE driving *lacZ* (b1-ARE-lacZ) would be active in RA-treated P19 cell monolayers. Surprisingly, b1-ARE-lacZ was poorly active in P19 EC cells when cultured in monolayer in the presence of RA (Fig. 1A-b). The transgene was efficiently activated only when RA-treated cells were also aggregated (Fig. 1A-d), suggesting that cell aggregation provides a signal required for HOXB1•PBX complexes to activate transcription.

An alternative explanation for these results is that the site of integration imposed constraints on the activity of the *Hoxb1* ARE. However, these experiments were done on populations of multiple clones representing many different sites of integration. Another possibility is that HOXB1, PBX, and MEIS/PREP proteins unexpectedly failed to accumulate upon RA treatment. This was not the case, as revealed by Western blot analysis (Fig. 1C). HOXB1 and PBX1 were both detected in P19 cell monolayers treated with RA at either of two concentrations. HOXB1 showed the most dramatic induction, while PBX1 was already present in untreated cells, and was modestly induced upon RA treatment. MEIS1 was also present before and after RA treatment (data not shown).

We hypothesized that in the absence of cell aggregation, HOXB1•PBX complexes could recruit HDACs to the *Hoxb1* ARE, thereby establishing a transcriptionally inactive condensed chromatin. To test this hypothesis, we treated the cells in monolayer with TSA, a histone deacetylase inhibitor, and measured reporter activity (Fig. 1B). As little as 20 nM TSA induced *lacZ* expression directed by the *Hoxb1* ARE, thereby circumventing the need for cell aggregation. In fact, TSA efficiently induced reporter gene expression in the absence of both RA and aggregation.

To investigate the effect of TSA on endogenous gene expression, we performed Western blot analysis on TSA-treated cultures. Figure 1C shows that TSA efficiently induced the expression of the endogenous *Hoxb1* gene, while PBX1 (Fig. 1C) and MEIS1 (data not shown) showed a moderate increase over pre-existing levels. Thus, TSA-treated cultures express all three homeoprotein families implicated in activation through the *Hoxb1* ARE. By contrast, TSA had no effect on a stably integrated control transgene (p1230) that lacks the *Hoxb1* ARE, establishing the specificity of this effect (Fig. 1B, inset). Together, these results suggest that HOXB1•PBX complexes recruit HDACs *in vivo* to repress transcription directed by the *Hoxb1* ARE. TSA treatment inhibits histone deacetylase activity, thereby inducing both the endogenous *Hoxb1* gene, and the b1-ARE-lacZ reporter.

The *Hoxb1* ARE used above is 150 bp long, and may contain binding sites for TSA-responsive transcription factors other than PBX or HOX proteins. To specifically test the response of HOX•PBX complexes to TSA, we transfected HEK293 cells with an artificial luciferase reporter, pML(5xHOX•PBX), driven solely by five HOX•PBX binding sites in front of a minimal promoter. pML(5xHOX•PBX) was repressed 5 fold relative to the parental vector pML lacking HOX•PBX binding sites (Fig. 2), again implicating HOX•PBX complexes in transcriptional repression. While pML was induced less than two fold by TSA, pML(5xHOX•PBX) was activated by 12 fold (Fig. 2), further supporting a role for HDACs in repression mediated by HOX•PBX complexes.

Over-expression of HOXB1, HOXA1 or HOXD4 enhanced the activation of pML(5xHOX•PBX) by TSA (Fig. 2) confirming the involvement of HOX proteins in this effect. By contrast, the TSA-response was dampened by overexpression of PBX1A (Fig.

2). Interestingly, deletion of the first 89 residues of PBX1A rendered the derivative protein highly TSA-sensitive, resulting in almost 100 fold activation of pML(5xHOX•PBX). We suggest explanations for this effect in the Discussion.

4.2 PBX is required for repression by HOX•PBX and for the response to TSA

The above results implicate HOX proteins in transcriptional activation through HOX•PBX binding sites, whereas PBX had a repressive effect. To assess the importance of PBX for repression and the TSA-response, we examined an independent reporter, pML(5xHOX), driven by monomeric HOX binding sites. In contrast to pML(5xHOX•PBX), pML(5xHOX) was not repressed in 293 cells and was not activated by TSA (Fig. 2). This result argues that PBX is required for the repression observed on pML(5xHOX•PBX) and for activation by TSA on this reporter. Reciprocally, HOX proteins cannot activate transcription efficiently in the absence of a PBX partner.

In a complementary test, we used derivatives of HOXA1 and HOXD4 harboring mutations in the conserved YPWM motif (A1 WM-AA and D4 WM-AA, respectively). This mutation has been previously shown not to affect the stability of HOXD4 (Rambaldi et al., 1994) and to abolish interaction between HOX and PBX proteins (Phelan and Featherstone, 1997; Phelan et al., 1995; Shanmugam et al., 1997; Shanmugam et al., 1999). As shown in Figure 2, while overexpression of HOXA1 or HOXD4 greatly enhanced the TSA effect on pML(5xHOX•PBX), this was abolished with A1 WM-AA and D4 WM-AA. These findings demonstrate that interaction of HOX with PBX is required for the TSA-response of pML(5xHOX•PBX). To explain these results, we

propose a model whereby physical interaction between HOX and PBX is required for association with coactivators and corepressors, respectively (see Discussion).

4.3 PBX1 interacts with class I HDACs

As shown above, PBX is required for TSA-sensitive repression mediated by HOX•PBX binding sites. The simplest explanation for this finding is that PBX directly interacts with one or more HDACs. To test this, we performed immunoprecipitation experiments using whole cell extracts from transfected 293 T cells. Flag-epitope-tagged HDAC1 and HDAC3, but not HDAC4, resulted in coprecipitation of PBX1 (Fig. 3A). This interaction is specific and shows a preference for the class I HDACs by HOX•PBX complexes. More stringently, rabbit polyclonal antibodies that specifically recognize PBX1 coprecipitated the endogenous HDAC1 and mSIN3B (Fig. 3B; lanes 1, 3). Interestingly, no interaction was observed with mSIN3A (Fig. 3B; lane 2) or with Mi2α or β (data not shown). However, as shown in Fig. 3D (lane 2), N-CoR, known to repress transcription in a mSIN3A complex (Nagy et al., 1997), coprecipitated with PBX1 *in vivo*. Thus, N-CoR/SMRT may associate with mSIN3B in the absence of mSIN3A.

To functionally characterize these interactions, we examined the effects of N-CoR and SMRT on pML(5xHOX•PBX). As shown in Fig. 3C, overexpression of either N-CoR or SMRT potentiated the repression observed on pML(5xHOX•PBX) in 293 T cells. Overexpression of an antagonist-bound estrogen receptor, in an attempt to titrate the endogenous levels of N-CoR/SMRT (Lavinsky et al., 1998), resulted in a partial relief of repression of pML(5xHOX•PBX). These data suggest that N-CoR/SMRT complexes are

recruited by HOX•PBX within the cell to exert significant repression effects on downstream targets.

4.4 Region 89-172 in the PBX1 N-terminus interacts with HDAC1

In PBX1, three N-terminal repression domains (corresponding to regions B, C and D in Fig. 4A) have been previously mapped (Lu and Kamps, 1996a). To directly characterize whether one of these repression domains recruits the HDAC complex, we generated multiple in-frame deletions in PBX1A (Fig. 4A) and examined *in vivo* association with HDAC1. Immunoprecipitation studies were carried out with extracts from 293 T cells cotransfected with plasmids expressing flag-tagged HDAC1 along with PBX1A-deletion derivatives. Following immunoprecipitation with anti-flag antibodies, the precipitates were analyzed by western analysis using polyclonal antibodies against PBX1 or anti-HA antibodies in the cases of Δ89-HA and 89-172-HA. Fig. 4B shows that the PBX1 N-terminus (ΔC232) is sufficient for HDAC1 binding.

Δ89 is highly responsive to TSA (Fig. 2), suggesting that the HDAC-interaction region in PBX1A is C-terminal to residue 89. As shown in Fig. 4C, Δ89-HA associated with HDAC1 and HDAC3 in whole cell extracts, mapping the region of interaction with HDAC1 to PBX1A regions C or D. Two deletions in region D were therefore tested and found to be dispensable for HDAC1 binding (Δ137-160 and Δ160-232, Fig. 4A, B). These data imply that region C is important for the recruitment of the HDAC complex by PBX1.

A deletion mutant of region C was not stable in mammalian cells. To address whether region C is sufficient for interaction with HDAC1, we used anti-HA antibodies

to immunoprecipitate a fusion protein containing the HA epitope fused in frame to residues 89-172 spanning region C of PBX1A. As seen in Fig. 4D, HDAC1 coprecipitated with HA-89-172 (lane 2) but not with an HA-control (lane 1). The above data indicate that while the region B repression mechanism is TSA-insensitive, region C recruits HDACs to repress transcription.

4.5 The HOXD4 activation domain binds the HAT-C/H3 domain of CBP

Treatment with TSA led to large increases in transcription from natural and artificial enhancers bearing HOX•PBX binding sites (Figs. 1, 2). Activation of pML(5xHOX•PBX) exceeded a simple loss of repression relative to pML (Fig. 2). These results show that TSA reveals a transcriptional activation function of the HOX•PBX heterodimer. Transcriptional activation is achieved through recruitment of coactivators by enhancer-bound proteins. One such co-activator is CBP. To assess its involvement in transcriptional activation by HOX•PBX complexes, we overexpressed CBP in 293 T cells. CBP stimulated expression from pML(5xHOX•PBX) ten- to twelve-fold, similar to the activation obtained by TSA treatment (Fig. 5A; lane 2). This result suggested that PBX, HOX or both, recruited CBP to target promoters.

We have previously characterized an activation domain in the proline-rich N-terminal half of HOXD4 (Rambaldi et al., 1994). We therefore tested whether the HOXD4 activation domain (HOXD4N, residues 3 to 141) could recruit CBP to a target promoter. Figure 5B (lanes 1, 2 and 3, black bars) shows that overexpression of CBP potentiates transactivation by a GAL4-HOXD4N fusion protein on the GAL4-responsive

reporter pML(5xUAS). In contrast, depletion of endogenous CBP by overexpression of the oncoprotein E1A neutralizes the coactivation effect seen with overexpressed CBP. E1A also inhibits the initial activation observed by HOXD4N (Fig. 5B, compare white bars in lanes 2 and 3 to black bars in lanes 1, 2 and 3). A deletion mutant of E1A that cannot bind CBP is unable to affect transcription significantly (gray bars in Fig. 5B). These results show that the transactivation function of HOXD4N is mediated by endogenous CBP. We also note that E1A interacts with the coactivator p300 through this same domain. None of our data excludes an interaction between HOX proteins and p300, in addition to CBP. Likewise, PCAF is expected to bind CBP in association with HOX (Yang et al., 1996b).

In vivo mapping studies were carried out to determine the respective domains of interactions between HOXD4 and CBP. A fusion of GAL4 to the HOXD4 N-terminus (GAL4-HOXD4N) but not to the C-terminus (GAL4-HOXD4C) coprecipitated with CBP, consistent with the N-terminal transactivation function of HOXD4 (Fig. 6A, lanes 1 and 2). To map the domains in CBP required for HOX binding, immunoprecipitation experiments were carried out with extracts from 293 T cells cotransfected with plasmids expressing GAL4-HOXD4N and one of four HA-tagged CBP domains: CBP-N, CBP-KIX, CBP-HAT-C/H3 or CBP-C (Fig. 6B). Analysis of the precipitates was carried out by western analysis with anti-HA antibodies. The four CBP domains used in this experiment were expressed at equivalent amounts in 293 T cells (data not shown). Fig. 6B shows that the HAT-C/H3 domains of CBP constitute the region of interaction with HOXD4N.

4.6 PKA signaling stimulates HOX•PBX promoters

The above results show that PBX and HOX proteins directly contact transcriptional corepressors and coactivators, respectively. What determines whether the HOX•PBX complex will have a net activating or repressive effect on gene expression? Our studies in P19 EC cells show that aggregation provides a signal that converts HOX•PBX complexes from repressors to activators. This conversion is dependent on cell aggregation. Among other possibilities, aggregation may increase the concentration of secreted growth factors, or allow presentation of surface-bound ligands to receptors on adjacent cells. Signaling *via* cyclic AMP (cAMP) second messenger is mediated by PKA. PKA has been implicated in the activation function of a number of transcription factors, including the homeoprotein PIT1. Given the known role of CBP in mediating the effects of PKA on transcriptional activation (Arias et al., 1994; Goldman et al., 1997), we tested the ability of PKA to convert HOX•PBX complexes from transcriptional repressors to activators.

Overexpression of the catalytic domain of PKA significantly stimulated pML(5xHOX•PBX) in 293 T cells (Fig. 5A). This effect was mediated through HOX•PBX binding sites since PKA had a minimal effect (2.6 fold) on pML lacking the HOX•PBX binding sites. This result suggests a link between the activation of the intracellular cAMP signal transduction pathway and the activity of HOX•PBX complexes.

We examined the impact of PKA signaling on transactivation of the GAL4-responsive reporter pML (5xUAS) by the GAL4-HOXD4N fusion protein. Figure 5B (lane 4) shows that PKA stimulated this reporter 500 fold in a HOXD4N-dependent

manner. The PKA stimulation requires CBP since depletion of endogenous CBP by overexpression of E1A inhibited this effect (lanes 4 and 5, white bars). Overexpression of PKA along with GAL4-HOXD4N and CBP-HA resulted in increased amounts of CBP coprecipitates with equivalent amounts of HOXD4N (Fig. 6A, lane 3). These data suggest that the recruitment of CBP by the activation domain of HOXD4 is facilitated in the presence of PKA. This further suggests a mechanism by which DNA-bound HOX•PBX complexes could be switched from repressors to activators through enhanced association with CBP.

5. DISCUSSION

Two observations suggested to us that HOX•PBX complexes may recruit transcriptional corepressors to target promoters. First, the *Hoxb1* ARE is inactive in RA-treated P19 cell monolayers despite the presence of HOXB1 and PBX1, but is activated in response to the HDAC inhibitor TSA (Fig. 1). Second, repression by multimerized HOX•PBX binding sites is likewise alleviated by TSA treatment (Fig. 2). Transcriptional activation through the *Hoxb1* ARE or multimerized HOX•PBX binding sites further suggested that HOX•PBX complexes recruit transcriptional coactivators. In support of this suggestion, a repression domain in the PBX1 N-terminus binds a corepressor complex containing class I HDACs in association with N-CoR/SMRT and mSIN3B (Fig. 3 and 4). Conversely, the proline-rich activation domain of HOXD4 binds the CBP coactivator. We provide additional evidence that the HOX•PBX complex can be switched from a repressor to an activator of transcription through the action of signaling cascades (Figs. 5, 6 and 7). Specifically, the HOX•PBX complex becomes a CBP-dependent transcriptional activator in response to PKA. Thus, the transcriptional activity of the HOX complex in a specific tissue at a given developmental stage may come under the control of signaling cues such as intracellular cAMP.

5.1 Repression of HOX•PBX targets is mediated by PBX-corepressor interactions

PBX1 has been previously shown to possess three repression domains in its N-terminus (Lu and Kamps, 1996a). Our results indicate that PBX1 represses transcription

through both HDAC-dependent and -independent mechanisms. We found that the first N-terminal repression domain of PBX1 (domain B) represses transcription in a TSA-resistant fashion. By contrast, the second N-terminal repression domain (within region C) associates with class I HDACs. Recently, others have shown that PBX1A binds N-CoR and SMRT through its C-terminus (Asahara et al., 1999). The set of PBX1A derivatives employed here does not refute this finding. Rather, the cumulative data suggest that PBX1A contains more than one docking site for corepressor complexes.

The corepressors N-CoR and SMRT are known to repress transcription in a mSIN3A complex (Nagy et al., 1997). In addition, SMRT has been shown to function in an HDAC3 complex (Guenther et al., 2000). The presence of mSIN3B and not mSIN3A in the corepressor complex recruited by PBX1 is a novel indication of an interaction between N-CoR/SMRT and mSIN3B.

Overexpression of wild type PBX1A inhibits TSA-mediated activation of a reporter bearing multiple HOX•PBX binding sites (Fig. 2, lane 5). By contrast, removal of the first 89 residues of PBX1A, or overexpression of HOX proteins, confers a strong TSA-response. Two non-exclusive explanations are possible. First, residues 1 to 89 of PBX1A may harbor a TSA-insensitive repression domain. This could be mediated by direct contact to a repressor, or indirectly through members of the MEIS/PREP family which bind PBX proteins through this N-temrinal domain (Chang et al., 1997b). This could explain the enhanced TSA-response with Δ1-89, but would not explain the dampened response with wild type PBX1A. Another explanation is that increased levels of PBX1A promote the formation of PBX•PBX homodimers at the target promoter. Such homodimers have been described in the literature (Calvo et al., 1999; Neuteboom and

Murre, 1997), and would be expected to form on the multimerized binding sites in pML(5xHOX•PBX). In theory, the PBX homodimer could compete with HOX•PBX heterodimers for DNA-binding, recruiting only co-repressors to the target promoter and thereby dampening the response to TSA. Deletion of the first 89 residues from PBX1A severely impairs homodimerization (K. Shanmugam and M.S.F., unpublished observations) without affecting heterodimerization with at least some HOX partners (Shanmugam et al., 1999). Thus, Δ1-89 would promote binding by HOX•PBX heterodimers at the expense of PBX homodimers, resulting in more efficient recruitment of coactivators.

Residues 1 to 89 of PBX1 are deleted in the oncoprotein E2A-PBX (Kamps et al., 1991). Thus, the increased transcriptional activation function, and concomitant oncogenicity of E2A-PBX, may be due to both the loss of a repression domain as well as the recruitment of HATs by the E2A activation domain (Cleary, 1991; Kamps et al., 1990; Massari et al., 1999). The HDAC1 binding domain in PBX1 (domain C) is retained in E2A-PBX. Consistent with this, TSA potentiates the activation observed by E2A-PBX (unpublished observations). Thus, treatment with TSA may potentiate B-cell transformation.

Domain C of PBX1 spans a short stretch of nine alanine residues and impinges on the conserved PBC-A and B domains. The PBC domains are highly conserved across species. By contrast, the alanine stretch is conserved in mammals and flies, but absent in the *C. elegans* CEH-20 protein. Monotonic alanine regions have been implicated in repressor function (Han and Manley, 1993a; Licht et al., 1994; Mailly et al., 1996),

however, at this time the highly conserved portions of PBC-A and B are equally plausible candidates for direct interaction with repressor complexes.

5.2 CBP modifies HOXD4 function and transduces PKA stimulation of HOX•PBX promoters

We have shown that the proline-rich activation domain of HOXD4 physically interacts with the HAT-C/H3 domain of the CBP coactivator. Interestingly, the interaction between HOXD4 and CBP seems to be conserved through evolution, since *Deformed*, the *Drosophila* orthologue of *Hoxd4*, has been shown genetically to interact with *Nejire*, encoding a transcriptional adapter belonging to the CBP/p300 family (Florence and McGinnis, 1998). A previous study has shown physical interaction between CBP and the N-terminus of HOXB7 (Chariot A, 1999). Using truncated versions of each protein *in vitro* and in transfections, their sites of interaction were mapped to the HOXB7 N-terminus and two regions in CBP including the C/H3 domain and the extreme C-terminus. Together with another study showing interaction between the N-terminus of the HOX-like protein PDX and the CBP C/H3 domain (Asahara et al., 1999), these findings suggest a common mechanism used by homeoproteins to activate transcription. To date, four *Hox* genes, namely *Hoxb1*, *Hoxa4*, *b4* and *d4*, have been shown to contain RAREs and AREs in their flanking regions (Gould et al., 1997; Huang et al., 1998; Langston and Gudas, 1992; Marshall et al., 1994; Morrison et al., 1996; Packer et al., 1998; Pöpperl and Featherstone, 1992; Pöpperl and Featherstone, 1993; Studer et al., 1994; Zhang et al., 2000). The HOX-interaction region in CBP centering on the C/H3 domain is different from the nuclear receptor interaction region (RID) (Chakravarti et al.,

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1996; Kamei et al., 1996). This suggests that one CBP molecule could simultaneously bind both retinoid receptor and HOX family members. This may result in synergistic recruitment of CBP to *Hox* gene promoters, thereby integrating the activities of retinoid receptors and HOX proteins.

Interactions between HOX and CBP can explain some of the phenotypes resulting from *Cbp* loss-of-function mutations. In man, the Rubinstein-Taybi syndrome (RTS) is caused by point mutations in the *Cbp* gene and is characterized by craniofacial deformations, broad thumbs, broad big toes, severe mental retardation and increased tumor incidence (Petrij et al., 1995). In the mouse, targeted disruptions of Cbp and p300 have revealed the importance of these cofactors in embryonic development (Yao et al., 1998). In *Drosophila*, mutations in *Cbp* cause embryonic lethality as well as pattern defects (Akimaru et al., 1997). Some of these defects are reminiscent of those caused by mutations in *Hox* genes (Krumlauf, 1994) and can be partly explained by the finding that CBP modifies HOX transcriptional activities.

Genetic and molecular studies in *Drosophila* have led to a model whereby the N-terminal activation domain of HOX proteins is masked due to direct or indirect contact with the HOX homeodomain (Li and McGinnis, 1999; Li et al., 1999). The model further suggests that this inhibition is relieved upon a conformational change provoked by cooperative DNA-binding of HOX with PBX. In this model, DNA-bound HOX monomers are repressors, while HOX•EXD (or HOX•PBX) heterodimers are activators. Our data are consistent with aspects of this model. First, TSA is able to activate a promoter driven by HOX•PBX dimer binding sites, but not one driven by HOX monomer binding sites. Second, mutations in the HOX YPWM motif that abrogate interaction with

PBX also abolish the TSA-response, even on HOX•PBX cooperative binding sites. Both of these observations would be expected if PBX is required to unmask the HOX activation domain thereby permitting interaction with CBP. However, the very fact that the HOX•PBX complex is responsive to TSA suggests a repressor function mediated by interaction with HDACs consistent with data reported here and elsewhere that PBX functions as a repressor and binds corepressors (Asahara et al., 1999; Lu and Kamps, 1996a).

In addition, we do not observe transcriptional repression by HOX monomers under our conditions. HOX monomer binding sites do not repress basal transcription (Fig. 2, compare pML to pML(5xHOX), and HOX mutants that are incapable of interacting with PBX partners do not behave as transcriptional repressors (Fig. 2) (Rambaldi et al., 1994). Rather, our data suggest that the HOX•PBX complex can act both as a transcriptional repressor and activator, depending on the cellular context (Fig. 7). We argue that this context can be influenced by cell-cell signaling, since aggregation is required to activate the *Hoxb1* ARE in RA-treated P19 cells. Monolayers of P19 cells can be induced down the neural pathway by combined treatment with forskolin, an activator of PKA signaling, and a factor secreted by cells resembling primitive streak mesoderm (Pruitt, 1994). This is consistent with a role for PKA in the activation of the *Hoxb1* ARE.

Our finding that CBP-HOX activation of downstream targets is significantly enhanced by PKA suggests a mechanism for conversion of HOX•PBX complexes from transcriptional repressors to activators. PKA was previously shown to be important for the transactivation of bovine CYP17 by PBX as well as the oncoprotein E2A-PBX via a cAMP-response sequence (CRS) (Ogo et al., 1995). The CRS in the promoter of CYP17

is very closely linked to a PBX-response sequence (PRS) that should accommodate cooperative binding by HOX•PBX *in vitro*. This suggests that the CRS response to PKA could be mediated by a HOX partner *via* CBP.

CBP contains a defined PKA phosphorylation site at serine 1772 shown to be important for mediating PKA-stimulated activation by the homeoprotein PIT1 (Xu et al., 1998). Our results likewise suggest that CBP phosphorylation by PKA is the signal transduction step required for HOXD4 to activate transcription in response to increased intracellular cAMP. We demonstrated increased association of the HOXD4 activation domain with CBP upon increased PKA signaling (Fig. 5A). How is this achieved? The levels of CBP are greatly increased in 293 cells expressing the catalytic subunit of PKA (unpublished observations). This increase may be sufficient to account for the greater association between HOXD4 and CBP upon PKA stimulation.

A role for PKA in HOX function in the embryo has not been clearly demonstrated. However, patterning by the hedgehog signaling pathway in flies and mice involves antagonizing the PKA pathway (Epstein et al., 1996; Noveen et al., 1996). Our results suggest that PKA may also impinge on patterning mediated by the HOX family. *Hox* genes are known to determine the morphogenetic outcome of cell signaling in fly imaginal discs (Percival-Smith et al., 1997). In *C. elegans*, genetic studies have shown that a HOX protein determines the developmental consequences of RAS signaling (Maloof and Kenyon, 1998). On theoretical grounds, HOX proteins were predicted to interpret cell signaling events in vertebrates as well (Davidson, 1991). Our results support this suggestion.

In summary, we have demonstrated that HOX•PBX can function as an activator or a repressor through differential interactions with coregulators. Moreover, we have shown that PKA serves as a signaling switch that converts HOX•PBX from repressors to activators, implying that cell signaling is an important determinant of the HOX•PBX function in the patterning of the animal embryo.

6. ACKNOWLEDGMENTS

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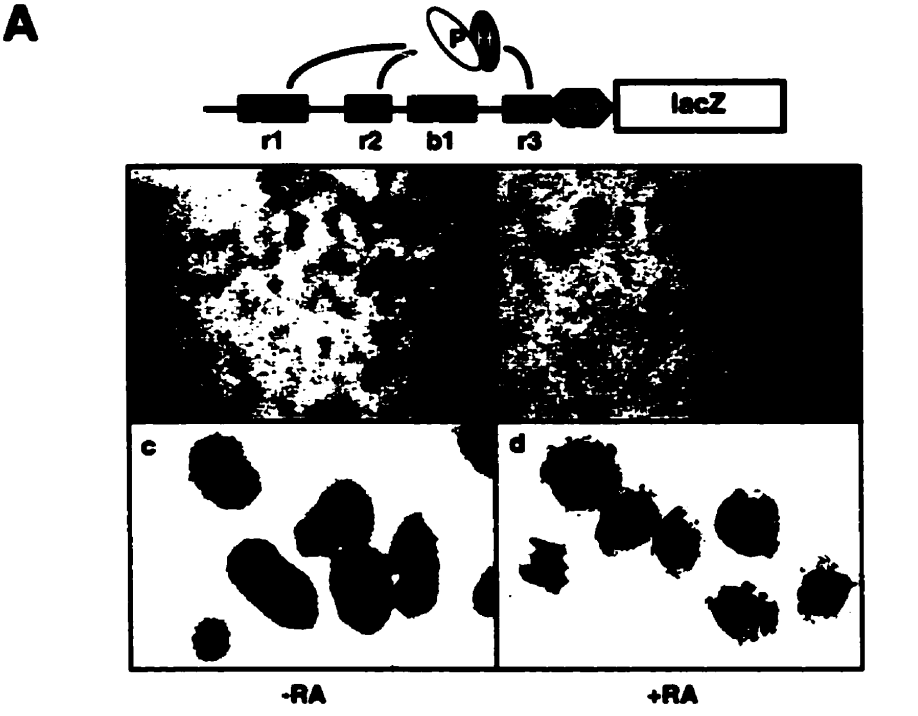
Figure. 1. TSA relieves the transcriptional repression of HOX•PBX–responsive enhancers.

(A) *Upper panel*: Representation of the b1-ARE-lacZ reporter used to stably transfect P19 cells. The black boxes r1, r2 and r3 represent three previously characterized HOX•PBX binding sites (72). The gray box b1 denotes "block 1", a region of homology conserved across species. Ovals labeled "P" and "H" denote the PBX•HOX complex. *Lower panel*: A stably-transfected transgene containing the *Hoxb1* ARE (b1-ARE-lacZ) was active in RA (3x 10^{-7}M)-treated P19 cells only if the cells were aggregated during RA exposure for 24h (1d) but not if the cells were kept cultured in monolayer (1b). P19 cell monolayers are shown in a and b, while cell aggregates are shown in c and d. Cells in b and d were treated with RA at 3 x 10^{-7} M for 24 h.

(B) TSA induces the activity of the b1-ARE-lacZ in monolayer in the presence and absence of RA. Liquid β galactosidase assays were carried out on P19 cells stably transfected with the b1-ARE-lacZ and cultured in monolayer. Monolayers were treated with either RA (3x10^{-7}M) or TSA (20 nM to 2 μM) or a combination of both for 24 h. Inset, similar assays were performed using a control transgene lacking the *Hoxb1* ARE (p1230).

(C) HOXB1 and PBX1 are induced in P19 cell monolayers in response to RA or TSA. Western analysis was performed using whole cell extracts from P19 cells cultured in monolayer in the absence or presence of treatment. RA was used at 3x10^{-7} M or 10^{-5} M and TSA was at 2 μM.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+RA

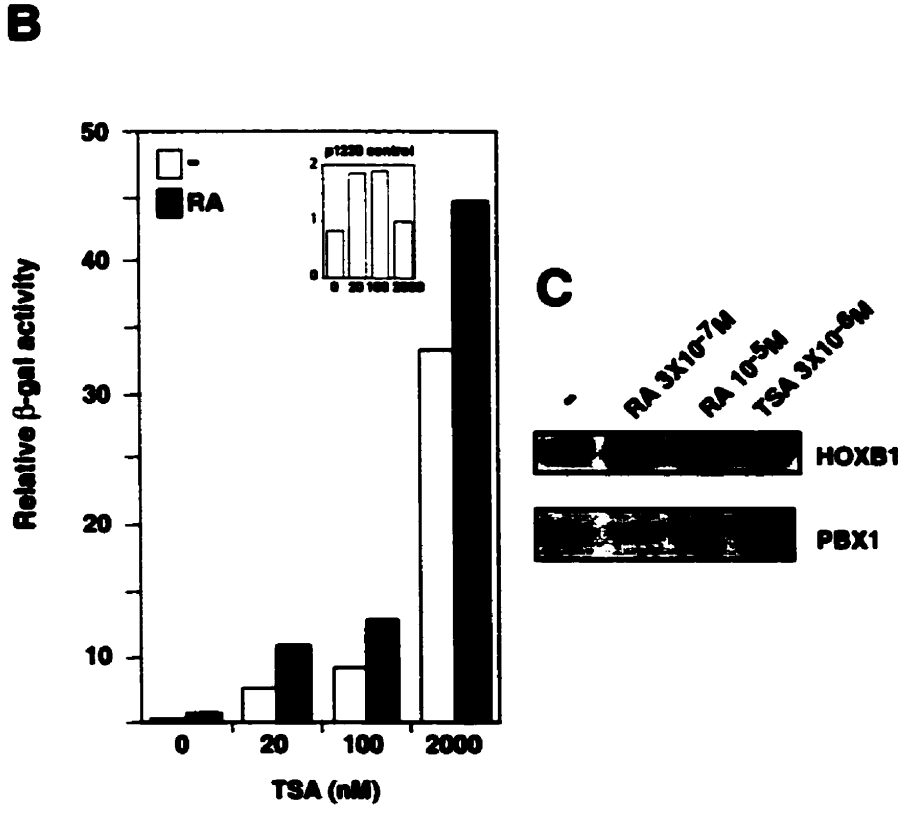
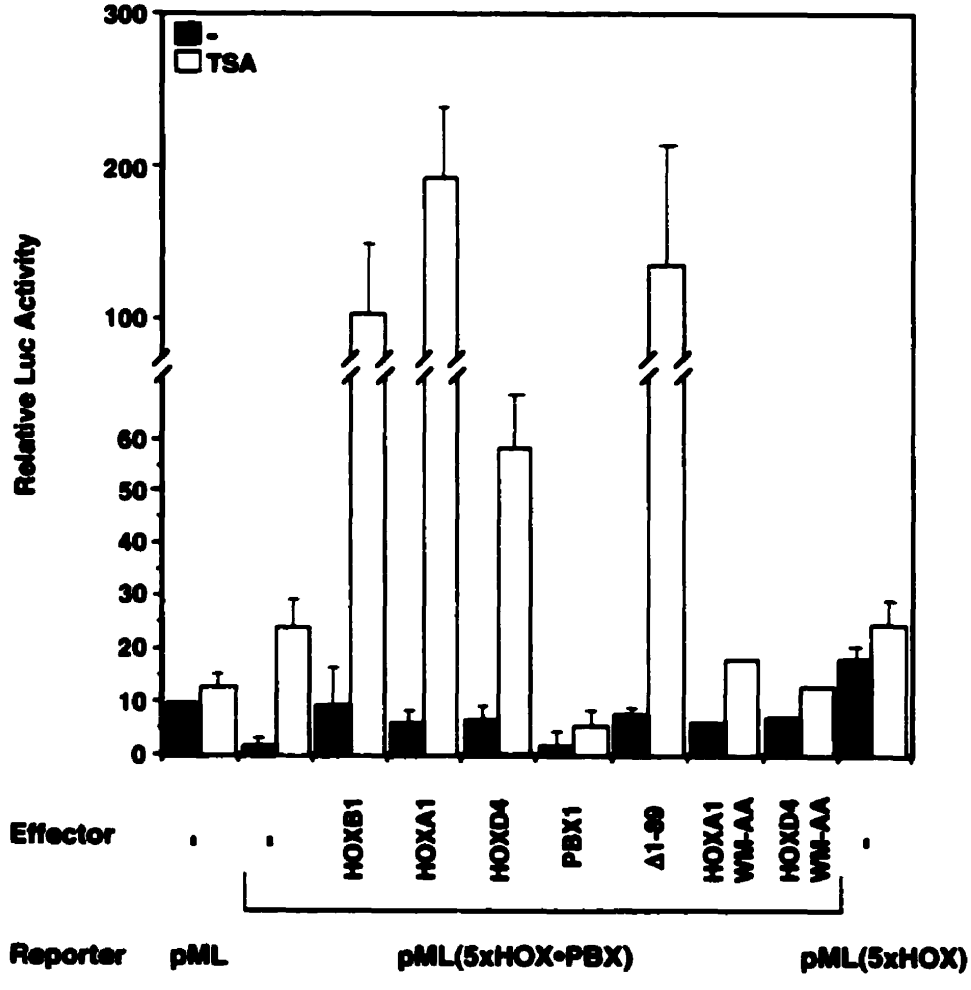

B
50
40
30
20
10
-
RA
Relative β-gal activity
0
20
100
2000
TSA (nM)
C
-
RA 3X10-7M
RA 10-5M
TSA 3X10-6M
HOXB1
PBX1

Figure. 2. PBX is required for the HOX•PBX-response to TSA.

pML(5xHOX•PBX), a reporter driven by five HOX•PBX binding sites, is repressed in transiently-transfected HEK 293 cells compared to pML which lacks HOX•PBX binding sites. pML(5xHOX•PBX) is significantly activated by TSA (2μM, 24 h) both in the absence or presence of over-expressed HOX and PBX1A proteins (black bars). Removal of residues 1-89 of PBX1A (Δ1-89) greatly increases reporter activation by TSA (lane 6). pML(5xHOX), containing five sites for monomeric HOX binding, is not repressed in 293 cells and is not further activated by TSA treatment (lane 7). Overexpression of HOXA1 or HOXD4, but not of A1 WM-AA or D4 WM-AA, transactivates transcription in the presence of TSA (lanes 3, 4, 8 and 9). All transfections were repeated at least three times in duplicate except for the D4 WM-AA experiment which was done once in duplicate.


300
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Effector
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HOXB1
HOXA1
HOXD4
PBX1
Δ1-99
HOXA1 WM-AA
HOXD4 WM-AA
-
Reporter
pML
pML(5xHOX•PBX)
pML(5xHOX)

Figure. 3. The HOX•PBX complex associates with class I HDACs *in vivo* and represses transcription in a mSIN3B/N-CoR/SMRT-dependent manner.

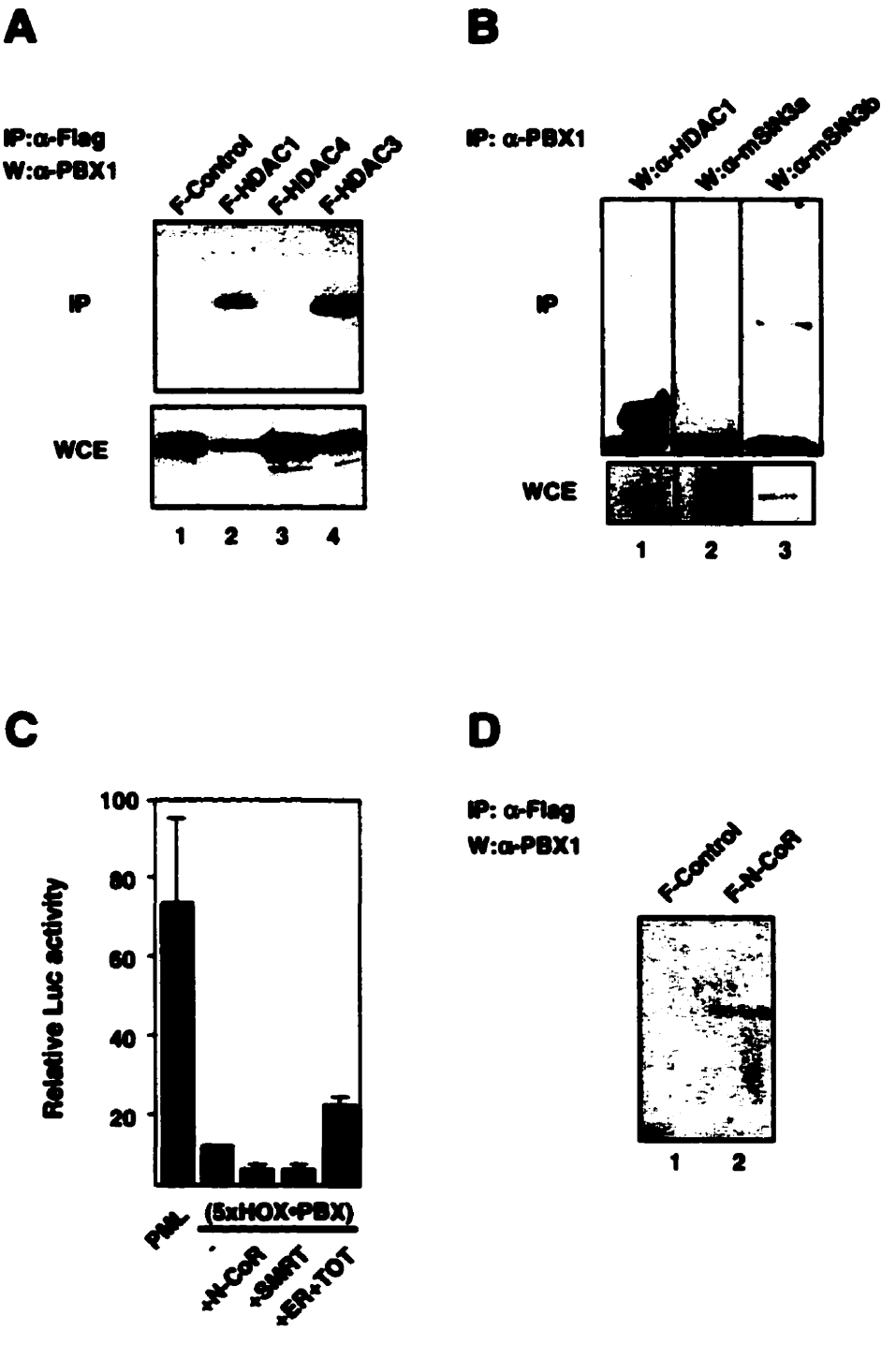
(A) PBX1 coprecipitates with class I HDACs (HDAC1 and HDAC3, lanes 2 and 4) but not with HDAC4 (lane 3) or from cells transfected with the empty flag vector (F-control) (lane 1). Immunoprecipitations were done with lysates from 293 T cells cotransfected with a plasmid expressing PBX1A along with that expressing flag-tagged HDAC1 (F-HDAC1), F-HDAC3, F-HDAC4 or F-control. Flag-tagged proteins were immunoprecipitated with M2 beads (Sigma) and the precipitates were eluted with flag peptides (Sigma) and analyzed by western blotting using rabbit polyclonal antibodies against PBX1 (Santa Cruz). "IP" and "WCE" denote immunoprecipitates and whole cell extracts used in western blot analysis. "W" denotes the antibody used in western analysis.

(B) Coprecipitation of endogenous HDAC1 and mSIN3B (but not mSIN3A) with rabbit polyclonal antibodies against PBX1. 293 T cells were transfected with a plasmid expressing PBX1A but not with plasmids expressing HDAC1, mSIN3B or mSIN3A. Immunoprecipitates with anti-PBX1 antibodies (IP: α PBX1) were analyzed in western blots with antibodies against HDAC1 (W:α-HDAC1), W:α-mSIN3a, and W:α-mSIN3b. The positions of bands corresponding to mSIN3b, HDAC1 and precipitating antibody (IgG) are indicated by arrows to the right of the blot.

(C) The repression of pML(5xHOX•PBX) in 293 T cells is exerted by N-CoR/SMRT-corepressor complexes. Overexpression of either N-CoR or SMRT further repressed pML(5xHOX•PBX). This repression can be partially relieved by sequestering

the endogenous N-CoR/SMRT with overexpressed estrogen receptor (ERα) bound to the estrogen antagonist TOT (see Materials and Methods).

(**D**) Immunoprecipitation of PBX1 from cells expressing flag-tagged N-CoR (F-N-CoR, lane 2) but not from cells transfected with the empty flag vector (F-control, lane 1).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F-Control
F-HDAC1
F-HDAC4
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W:α-HDAC1
W:α-mSin3a
W:α-mSin3b
IP
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C
Relative Luc activity
100
80
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40
20
PML
(5xHOX+PBX)
+N-CoR
+SMRT
+ER+TOT
D
IP: α-Flag
W:α-PBX1
F-Control
F-N-CoR
1 2

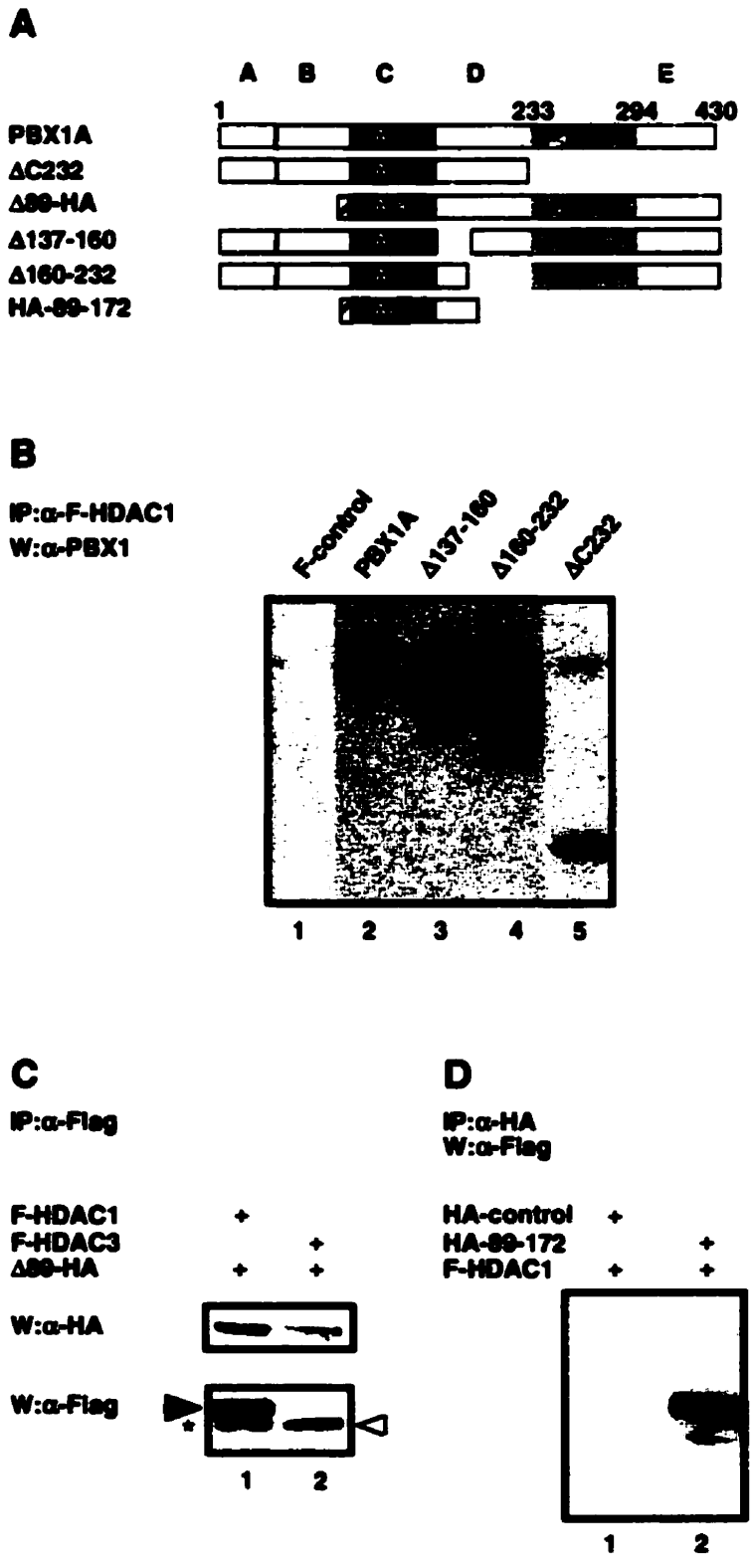
Figure. 4. Region C of PBX1A is responsible for the interaction with HDAC1.

(A) Schematic representations of wild type PBX1A and PBX1A deletion mutants. The subdivision of the PBX1A N-terminus into four domains labeled A, B, C and D is after (Lu and Kamps, 1996a). The striped rectangle indicates the position of the HA-tag in Δ1-89 and in HA-89-172.

(B) The PBX1A N-terminus interacts with HDAC1. Binding studies similar to those described in Figure 3A were carried out for PBX1A and PBX1A mutants with flag-tagged HDAC1 immunoprecipitated on M2 beads and eluted with flag peptide. Anti-PBX1 antibodies were used for the western analysis.

(C) Regions A and B of PBX1A are dispensible for interaction with HDAC1 and 3. Similar experiment as in (B) except that the Δ1-89 mutant was tagged with the HA epitope and was recognized in western by anti-HA antibodies (Babco). The black arrowhead indicates HDAC1, the white arrowhead indicates HDAC3 and the asterisk indicates an HDAC1 degradation product.

(D) HDAC1 coprecipitates with region 89-172 of PBX1A. Cells were transfected with a vector expressing flag-tagged HDAC1 and either an empty HA vector (HA-control) or one expressing HA-tagged region 89-172 of PBX1. IP experiments were carried out with anti-HA antibodies, and anti-flag antibodies were used in western analysis.


A
A B C D E
1 233 294 430
PBX1A
ΔC232
ΔB9-HA
Δ137-160
Δ160-232
HA-89-172
B
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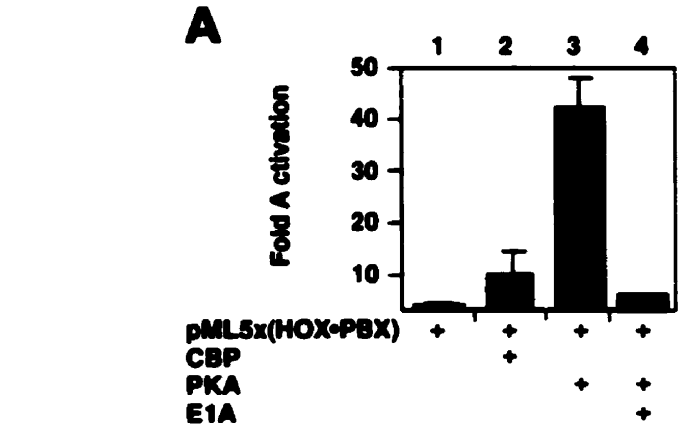
W:α-PBX1
F-control
PBX1A
Δ137-160
Δ160-232
ΔC232
1 2 3 4 5
C
IP:α-Flag
F-HDAC1 + -
F-HDAC3 - +
ΔB9-HA + +
W:α-HA
W:α-Flag
1 2
D
IP:α-HA
W:α-Flag
HA-control + -
HA-89-172 - +
F-HDAC1 + +
1 2

Figure. 5. CBP enhances the transactivation potential of HOX•PBX complexes and is required to transduce PKA signaling.

(A) pML(5xHOX•PBX) is activated by overexpression of CBP in 293 T cells and is super-activated by the catalytic domain of PKA. Activation by PKA is inhibited by overexpression of E1A.

(B) A fusion of the N-terminus of HOXD4 to the GAL4 DNA-binding domain (GAL4-HOXD4N) is able to transactivate transcription from a heterologous promoter driven by 5X GAL4 binding sites (pML(5xUAS)) (lanes 1 and 2, black bars). CBP potentiates the transactivation function of HOXD4N on this reporter (lane 3, black bar) in a manner sensitive to E1A (white bar) but not E1A ΔN (gray bar), a mutant deficient in CBP binding. PKA stimulates HOXD4N transactivation in a CBP-dependent manner (lanes 4 and 5).


A
1
2
3
4
50
40
30
20
10
Fold A ctivation
pML5x(HOX•PBX)
CBP
PKA
E1A

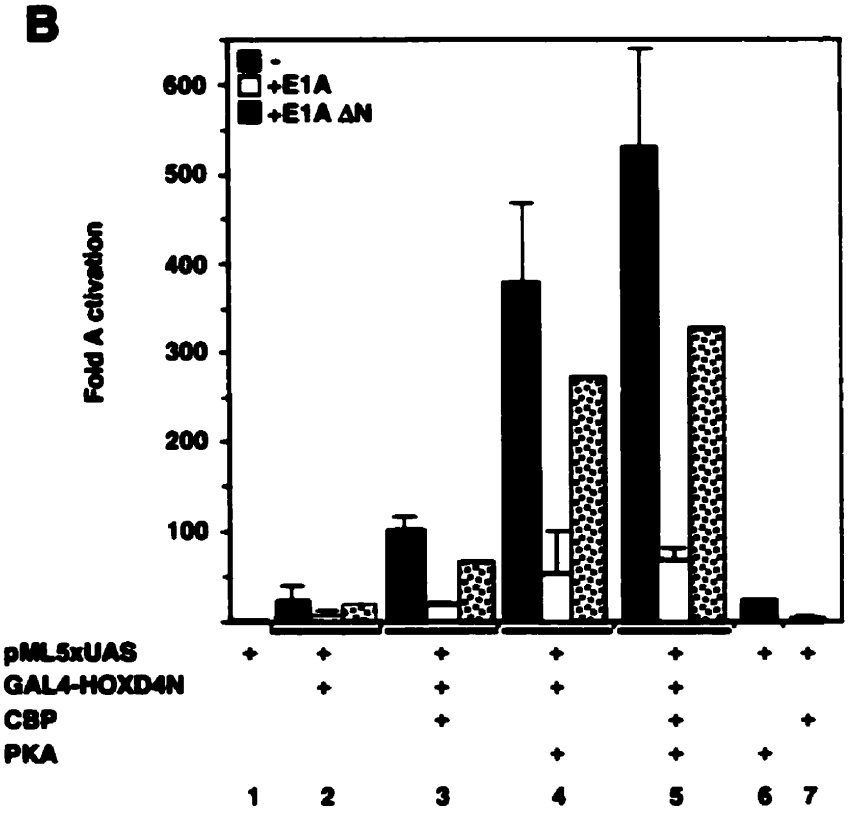
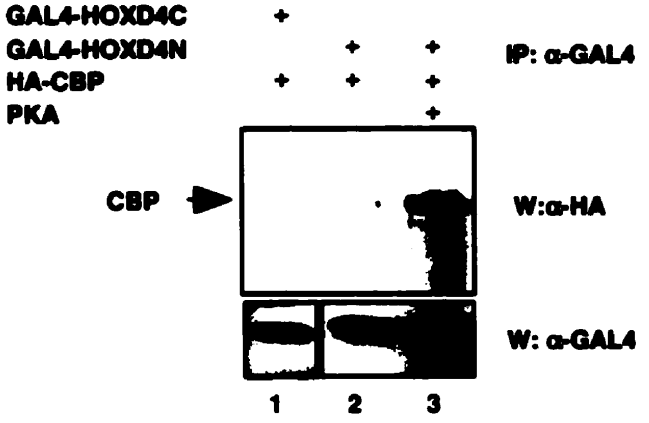

B
-
+E1A
+E1A ΔN
600
500
400
300
200
100
Fold A ctivation
pML5xUAS
GAL4-HOXD4N
CBP
PKA
1
2
3
4
5
6
7

Figure. 6.

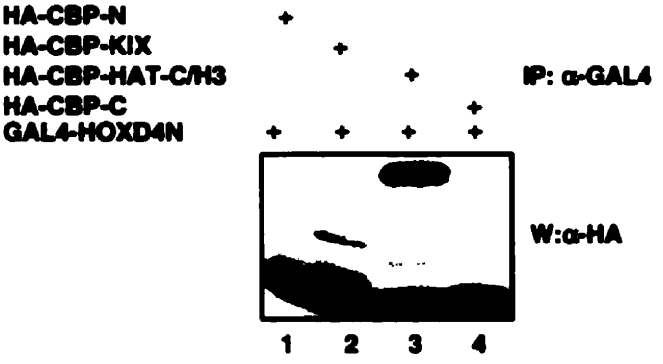
(A) Interactions between the HOXD4 N-terminus and CBP. GAL4-HOXD4N or GAL4-HOXD4C were immunoprecipitated with antibodies against the GAL4 DBD. Interaction with HA-tagged CBP (HA-CBP) in the presence or absence of overexpressed PKA was assessed by western analysis using anti-HA antibodies.

(B) The HOXD4 N-terminus coprecipitates with the CBP HAT-C/H3 domains. Immunoprecipitation studies were performed on whole cell extracts from 293 T cells cotransfected with GAL4-HOXD4N along with four HA-tagged domains of CBP: HA-CBP-N (amino acids 1-460), HA-CBP-KIX (amino acids 460-662), HA-CBP-HAT-C/H3 (amino acids 1450-1903) or HA-CBP-C (amino acids 2040-2170). IP was performed with antibodies against the GAL4 DBD and the CBP domains were detected by western analysis using anti-HA antibodies. The schematic representation of the CBP protein is after Chariot *et al.* (Chariot A, 1999).

A


GAL4-HOXD4C
GAL4-HOXD4N
HA-CBP
PKA
IP: α-GAL4
CBP
W:α-HA
W: α-GAL4
1
2
3

B


HA-CBP-N
HA-CBP-KIX
HA-CBP-HAT-C/H3
HA-CBP-C
GAL4-HOXD4N
IP: α-GAL4
W:α-HA
1
2
3
4

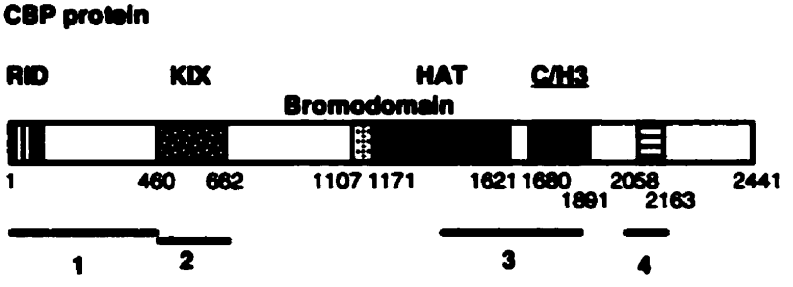
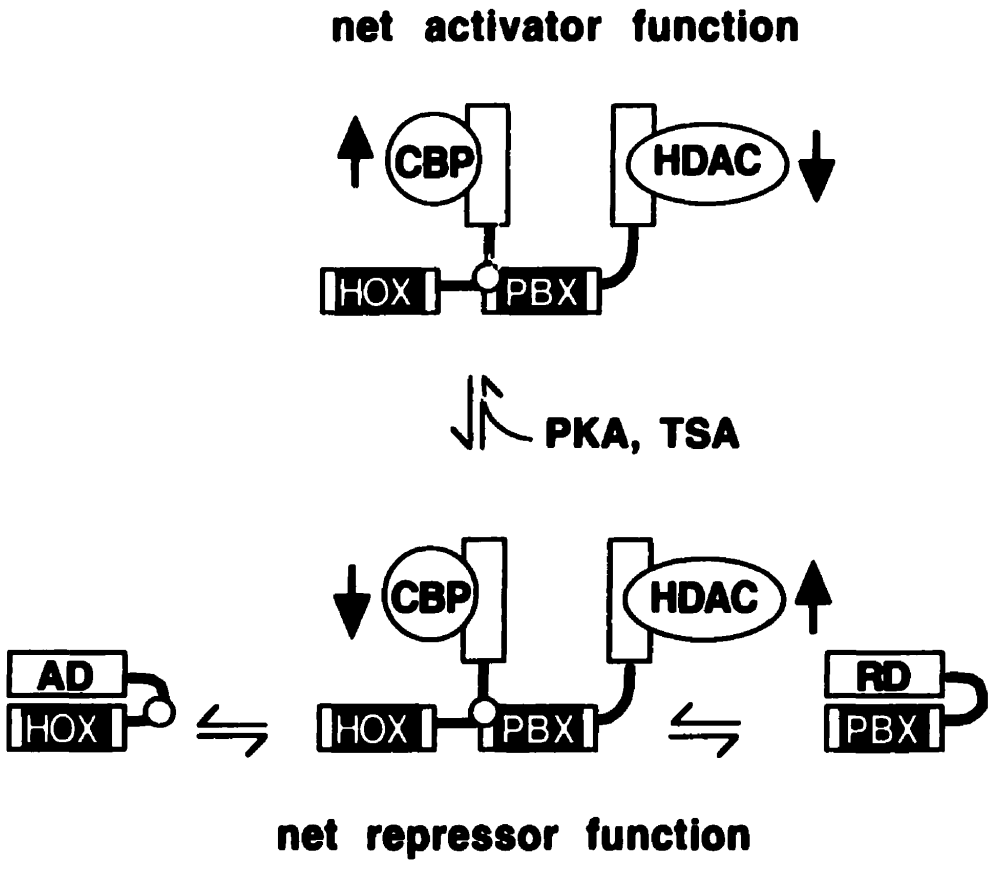

CBP protein
RID
KIX
HAT
C/H3
Bromodomain
1
460
662
1107
1171
1621
1680
1891
2058
2163
2441
1
2
3
4

Figure. 7. A model for activation and repression by HOX•PBX complexes.

The N-terminal activation and repression domains of HOX and PBX proteins are believed to make intramolecular contact with their respective homeodomains (Calvo et al., 1999; Li and McGinnis, 1999; Li et al., 1999; Neuteboom and Murre, 1997; Saleh et al., 2000a). Heterodimerization on cooperative sites on DNA, and perhaps additional interactions with members of the MEIS/PREP family, expose the HOX and PBX N-termini, thereby freeing them for interaction with coactivators and corepressors like CBP and HDAC1 and 3. Under some cellular contexts, the net activity of bound corepressors exceeds that of the activators (bottom portion of figure, "net repressor function".) However, in response to enhanced PKA signaling or P19 cell aggregation, increased coactivator and/or decreased corepressor function shifts the balance towards net activation (top portion of figure.) This could be accomplished by an increase in the amount of coactivator or by increased affinity for the HOX N-terminus. In parallel, decreases in the amount or affinity of corepressor for PBX could contribute to the switch. Treatment with TSA would exert the same overall effect by inhibiting bound HDACs. The model is simplified, and does not exclude other possible interactions. The black vertical arrows denote increases or decreases in HAT or HDAC activity. AD, HOX activation domain; RD, PBX repression domain C; black box, homeodomain; small white circle, HOX YPWM motif.


net activator function
CBP
HDAC
HOX
PBX
PKA, TSA
CBP
HDAC
AD
HOX
HOX
PBX
RD
PBX
net repressor function

CHAPTER IV

GENERAL DISCUSSION

Specific target recognition by HOX proteins is essential for their differential roles during body patterning. Specificity of DNA-binding by HOX is to a large extent conferred by interactions with DNA-binding partners of the PBX and MEIS families (for review see Mann and Affolter, 1998). The availability of such cofactors in the nucleus is one mechanism by which HOX functions could be regulated in a tissue-specific manner. We and others have shown that MEIS proteins regulate PBX nuclear localization (chapter 2) (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Rieckhof et al., 1997; Saleh et al., 2000a). In the limb bud, PBX is nuclear in only the proximal but not the distal cells, a distribution revealed to be important for limb pattern formation (Capdevila et al., 1999; Casares and Mann, 1998; Gonzalez-Crespo et al., 1998; Mercader et al., 1999). A question arises from these studies: Are HOX proteins in the distal limb not functional, or do they regulate a different subset of targets in the absence of DNA-binding partners? Genetic studies from one report indicated that in the absence of PBX, HOX proteins function as transcriptional repressors, and that the role of PBX is to switch HOX transcriptional function from repression to activation (Pinsonneault et al., 1997). Detailed analysis of the mechanisms of transcriptional regulation by HOX•PBX is thus required to test this hypothesis and is presented in chapter 3 (Saleh et al., 2000b). In this General Discussion, I address some of the unanswered questions related to the control of HOX functions by the nuclear localization of its cofactors and of HOX•PBX regulation of transcription in response to cell signaling. In addition, I propose relevant future experiments for better understanding of the role of MEIS in PBX's nuclear export, the function of PBX in HOX-target regulation, the function of MEIS in transcriptional

regulation, the role of signaling pathways in HOX regulation and the effects of post-transcriptional modifications on the functions of HOX•PBX complexes.

1. Examination of PBX nuclear export and the role of MEIS/PREP1 in this process

The nuclear export of PBX and EXD has been shown to be LMB-sensitive and thus mediated by the CRM1/exportin 1 nuclear export receptor (Abu-Shaar et al., 1999; Berthelsen et al., 1999). No NES was proven to exist in PBX and EXD and the region that mediated nuclear export was mapped to different domains in the two proteins. This suggests that PBX/EXD could possibly be carried to the cytoplasm by an adapter protein with a LMB-sensitive NES of its own. To test this hypothesis, *in vitro* binding assays, such as GST pull-down experiments, can be simply performed to detect direct interaction between PBX/EXD and CRM1/exportin 1. Adapters involved in the export of nuclear proteins have been described and include IκB (Arenzana-Seisdedos et al., 1997; Johnson et al., 1999) or the 30 KDa protein 14-3-3 (that exists in nine isoforms in mammals) (Liu et al., 1995; Xiao et al., 1995). 14-3-3 has been demonstrated to mediate the nuclear export of Cdc-25 (Kumagai and Dunphy, 1999; Lopez-Girona et al., 1999) and more recently of HDAC4 (Grozinger and Schreiber, 2000; Wang et al., 2000), and has been suggested to function as an attachable NES. The observation that, in our system, one 14-3-3 isoform did not interact with PBX in an immunoprecipitation experiment (data not shown) does not preclude the possible role of the other isoforms in this process. *In vitro* binding assays or immunoprecipitation experiments can be used to detect any association between the eight other 14-3-3 isoforms and PBX. In the case of negative interactions,

each of the two domains mapped by Abu Shaar *et al.* and Berthelsen *et al.* could be used in a yeast two hybrid assay to identify interacting proteins that may function in PBX nuclear export (Abu-Shaar et al., 1999; Berthelsen et al., 1999). Similar experiments could be performed to test the possibility of the presence of a cytoplasmic retention factor that would anchor PBX in the cytoplasm in the absence of MEIS/PREP1. Evidence from our laboratory may suggest the presence of such a cytoplasmic retention factor in the regulation of PBX nuclear availability (unpublished data). Identification of additional PBX or MEIS/PREP1 interacting proteins that are involved in the control of PBX subcellular localization may be crucial for our understanding of the control of HOX functions by PBX. These factors could be tissue-specific and could interact differently with the different MEIS family members or with PREP1 to result in differential PBX nuclear localization in different tissues.

2. Deletion of PBX region 1-89 *in vivo*

Region 1-89 within the PBC-A domain of PBX has been shown to be multifunctional. It mediates PBX's interaction with MEIS/PREP1 (Berthelsen et al., 1998b; Chang et al., 1997b; Knoepfler et al., 1997), represses transcription of HOX•PBX downstream targets in a TSA-insensitive manner (Saleh et al., 2000b) and has been recently demonstrated to be required for PBX's but not EXD's nuclear export (Berthelsen et al., 1999). Interestingly, this region is deleted in the oncoprotein E2A-PBX (Kamps et al., 1990; Nourse et al., 1990). Understanding the role of this region *in vivo* may thus provide insights towards the elucidation of the mechanisms of oncogenesis by E2A-PBX.

This could be achieved by deleting residues 1 to 89 *in vivo*, using gene targeting as a tool to replace the mouse *Pbx1* gene by a cDNA encoding PBX Δ1-89 (see appendix, Fig. 1). Deletion of region 1-89 in PBX was shown by Berthelsen *et al.* to render the derivative protein constitutively nuclear in S2 insect cells (Berthelsen et al., 1999). Our results in chapter 2 contradict these findings and show that PBX1 Δ1-89 is cytoplasmic in 60 % of the cells scored (Saleh et al., 2000a). These contradictory results could be due to variable factors including different S2 cell origins or even different cell culturing protocols. In mammalian Cos-7 cells, both wild-type PBX1 and PBX1 Δ1-89 are in the nucleus. Deletion of these residues would remove the NES and possibly induce a conformational change exposing the NLS in the PBX HD. Nonetheless, independence from MEIS/PREP1 for nuclear localization could be accomplished, for example, by a post-translational modification of PBX1A that inactivates the NES or alters the conformation of the N-terminus to expose the NLS in the HD, or by loss of a cytoplasmic retention factor.

To resolve this controversy, it would be reasonable to examine the subcellular localization of the *in vivo*-deleted PBX protein, in the mouse. The "knock-out/knock-in" mouse would show variable phenotypes depending on the subcellular localization of PBX Δ1-89. If PBX Δ1-89 is constitutively nuclear, as has been reported by Berthelsen *et al.* (Berthelsen et al., 1999), one would be able to examine the effects of nuclear PBX in tissues where PBX is normally cytoplasmic, as in the distal limb. In addition, one would observe the effects of the loss of MEIS/PREP1 interaction on the regulation of PBX•MEIS/PREP1 and HOX•PBX•MEIS/PREP1 downstream target genes. This mutant background would also allow the analysis of the modified transcriptional function of

PBX that may lead to aberrantly regulated HOX•PBX Δ1-89 target genes. In contrast, if PBX Δ1-89 is mainly cytoplasmic, as revealed in our study and in Abu Shaar *et al.* (Abu-Shaar et al., 1999; Saleh et al., 2000a), the mutant mouse would present phenotypes that are either equivalent or less severe than those observed in the embryonic lethal *Pbx*$^{-/-}$ mutant. It would be interesting to analyze the defects that could result in such a hypomorph especially if the mice are viable. This experiment is essential for the understanding of the multiple functions of PBX in development and cancer.

3. The role of MEIS/PREP1 in transcriptional regulation

Regulation of the *Hoxb2* r4 enhancer activity in the mouse embryo hindbrain has been recently shown, by transgenic analysis, to be dependent on the formation and binding of HOX•PBX•MEIS/PREP1 trimeric complexes (Ferretti et al., 2000; Jacobs et al., 1999). Deletion or mutation of the binding site of any component of the trimer leads to the inhibition or misregulation of the enhancer activity. This suggests that MEIS/PREP1 recognition of the enhancer is essential to mediate transcriptional activation in r4. We have dissected in chapter 3 the mechanisms of repression and activation by HOX•PBX complexes (Saleh et al., 2000b). However, we did not address the role of MEIS/PREP1 in these processes. We performed immunoprecipitation experiments to demonstrate differential recruitment of corepressors and coactivators by PBX and HOX, respectively, to regulate transcription. The possibility of MEIS/PREP1 proteins being immunoprecipitated in our assays in association with the coregulator complexes is worth consideration. However, mapping studies demonstrating interactions between specific domains in PBX and HOX, to which MEIS/PREP1 do not bind, and the

coregulators (region C in PBX with HDAC1 or HOXD4 N with the HAT-C/H3 domain of CBP) preclude the presence of MEIS/PREP1 as mediators of such interactions. Nevertheless, MEIS/PREP1 could be in direct association with other components of the coregulator complexes or with proteins of the general transcription machinery or the SRB mediator complex, contributing as such to transcriptional regulation. Characterization of the effector domains in MEIS/PREP1 proteins and investigation of their direct binding to members of the transcriptional regulatory complexes would therefore be required for full understanding of HOX functions. Deletional analysis coupled with generation of chimeric proteins between various regions from MEIS/PREP1 and a heterologous DNA-binding domain (DBD), such as GAL4 DBD, would determine the presence or not of activation or repression domains in MEIS/PREP1. *In vitro* binding assays, such as GST pull-down experiments, would be subsequently used to determine direct binding of MEIS/PREP1 with an array of proteins (within the coregulator complexes or the general transcription machinery) to be tested. These experiments would allow us to know what protein binds to what, starting from the HOX•PBX•MEIS-responsive enhancer and ending at the POLII holoenzyme.

4. Signaling pathways in the regulation of HOX functions

Very little is known about the signaling pathways that function upstream of the mammalian HOX proteins to modulate their activities. In *Drosophila*, signaling molecules including fibroblast growth factors (FGFs), members of the transforming growth β (TGFβ) family such as decapentaplegic (DPP), wingless (WG) and hedgehog (HH) have been implicated by genetic studies in HOX regulation. However, the direct

link between these pathways and the transcriptional regulation of downstream target by HOX proteins has not been clearly established. We show in chapter 3 that PKA signaling enhances the transcriptional activation function of HOX•PBX complexes and stimulates HOX•PBX-responsive enhancers in a HOX-activation-domain-dependent manner. We also show that the coactivator CBP recruited by the HOX protein activation domain transduces the PKA signaling (Saleh et al., 2000b). Recently, PKA signaling has been demonstrated to result in the phosphorylation of some HOX proteins, modifying their DNA-binding ability (Berry and Gehring, 2000; Dong et al., 1998). Therefore, it would be interesting to investigate the substrate of PKA in the HOX•PBX•coactivator complex and study the effect of PKA phosphorylation on protein-protein or protein-DNA interactions. Our results revealed an increased association between CBP and HOX activation domain in response to PKA, suggesting an effect of phosphorylation on protein-protein interactions. CBP has been previously shown to possess a PKA consensus phosphorylation site, and to be phosphorylated by PKA on serine 1772 (Xu et al., 1998 and references therein). This implicates CBP as a PKA substrate in the HOX•PBX•coactivator complex but does not exclude the possible phosphorylation of either PBX or HOX proteins. PBX has been implicated in the cAMP-dependent activation of the *CYP 17* gene, however it has not been shown to be phosphorylated by PKA (Bischof et al., 1998a; Ogo et al., 1997a; Ogo et al., 1995; Ogo et al., 1997b). On the other hand, previous work from our laboratory indicated a possible phosphorylation of HOXD4 by PKA *in vitro*. Further work is required to correlate HOXD4 phosphorylation by PKA *in vivo* with transcriptional activation (as in the RA-treated P19 aggregates). Transactivation by PKA could thus result from the phosphorylation of

components of the HOX•PBX complex or its associated coregulators, that may lead to better DNA-binding by HOX•PBX or a more stable interaction with the general transcription machinery. A stronger association with the PIC converts the promoter of a downstream target into a stronger promoter, favoring initiation of transcription. The role of PKA in the regulation of HOX activity provides a direct link for HOX proteins to sense their cell's environment and regulate their targets accordingly. This comes in a perfect agreement with a former observation that predicted HOX proteins to interpret cell signaling events in vertebrates (Davidson, 1991).

Cell aggregation is another determinant of HOX activity (chapter 3). P19 cells are EC cells that can be differentiated towards the neural or mesodermal pathways following aggregation and treatment with RA or dimethyl sulfoxide (DMSO), respectively (Rudnicki and McBurney, 1987). The differentiation of these cells mimics early embryonic development and is thus a suitable system to study HOX functions. RA-treated P19 cells show increased levels of HOX proteins and their cofactors (Ferretti et al., 2000; Knoepfler and Kamps, 1997; LaRosa and Gudas, 1988; Oulad-Abdelghani et al., 1997). However, despite the presence of these proteins in the cell, the *Hoxb1* autoregulatory element (ARE) is repressed when the cells are cultured in RA-treated monolayer. Aggregation of the cells induces the *Hoxb1* ARE and switches HOX•PBX complexes from repressors to activators of transcription (Saleh et al., 2000b). What are the signaling pathways downstream of cell aggregation? Cotreatment of P19 cells in monolayer with RA and forskolin (an activator of PKA signaling) did not induce the *Hoxb1* ARE (data not shown), suggesting that PKA signaling can not substitute for cell aggregation in this assay. Interestingly, a previous report implicated two signaling

pathways in P19 neural induction. These included PKA signaling, and a signal from cells resembling primitive streak mesoderm (Pruitt, 1994). It would be helpful to use this system to unravel the components of the signaling pathway upstream of HOX function. One could aggregate the cells, then use inhibitors of PKA signaling to study whether PKA signaling is induced by cell aggregation, and whether the PKA inhibitors would inactivate the *Hoxb1* ARE enhancer function.

One could also investigate the role of more upstream candidates in the induction of cell aggregation activating pathways, such as cell adhesion molecules (CAM), the calcium-dependent cadherin family or other cell surface molecules that initiate the cell contact. This could be done using the *Hoxb1* ARE P19 stable aggregates (described above), and subsequent inhibition of the cell adhesion molecules by immunodepletion (monoclonal antibodies against the cell adhesion molecules) or by blocking the receptors with inhibitory peptides harboring the cell adhesion recognition sequence. Would inhibition of the cell surface receptors have an effect on the activity of the *Hoxb1* ARE enhancer? It is interesting to note that genes coding for cell surface molecules are among the rare HOX-downstream targets identified to date (Edelman and Jones, 1995; Gould and White, 1992; Graba et al., 1992; Jones et al., 1992). One can speculate a positive feedback loop whereby cellular aggregation mediated by cell surface receptors activate HOX proteins, and HOX proteins in turn induce such cell surface receptor genes to stabilize or maintain the cell aggregation.

5. The role of acetylation in the regulation of HOX•PBX functions

Phosphorylation of HOX proteins by PKA may constitute a regulatory step in activation of target genes, as mentioned in the previous section. Other post-transcriptional modifications that may alter HOX function include protein acetylation. The association of HOX proteins with CBP and P/CAF (chapter 3 and data not shown) in transcriptional activation directed us to investigate whether these HATs acetylate HOX and PBX proteins *in vivo*. We show that while HOXD4 is only acetylated by P/CAF, the PBX HD is acetylated by both P/CAF and CBP (see appendix, Fig. 2). *In vitro* acetylation of HOXD4 and PBX HD led to altered DNA-binding abilities by the modified proteins, as revealed from EMSA analysis. Acetylation of HOXD4 inhibited its monomeric DNA-binding ability, while that of PBX HD improved the binding by the HD to DNA (see appendix, Fig. 3). Several questions arise from these observations. First what is the consequence of acetylation of both proteins *in vivo* on the formation of a cooperative complex? Second, which lysines are acetylated in the two proteins, and why is PBX acetylated by two HATs?

CBP and P/CAF acetylate residues in the purified PBX HD. As mentioned in chapter 2, the PBX HD possesses two NLS that are rich in lysines. Nevertheless, we did not find any correlation between acetylation and PBX nuclear localization (data not shown). Our assay, however, examined the effects of inhibition of HDAC activity (by treatment of the cells with TSA) or of overexpression of CBP and PCAF. More stringently, the lysine residues within the NLS need to be mutated to arginines, to inhibit acetylation of the NLS without disrupting its structure, and the unacetylable NLS would then be tested for nuclear localization function.

In the case of HOXD4, the recombinant protein used in the *in vitro* acetylation assays had a deletion of HOX N-terminus up to the YPWM motif, and most of the lysine residues in this protein are located in the HD, except for two conserved lysines that follow the YPWM KK. Mutagenesis of all the lysines in HOXD4 was performed, and identification of the lysine that gets acetylated would provide information about the *in vivo* function of this modification in relation to HOX regulation. It would be interesting to correlate acetylation of HOX•PBX to transcriptional regulation (either activation or repression). This could be achieved using the *Hoxb1* ARE enhancer in the P19 system. In cases of activation, one could determine the acetylation status of HOX or PBX proteins and their ability to bind to DNA by using a modified version of the ChIP (chromatin immunoprecipitation) assay, in which antibodies raised against unacetylated or acetylated lysines of either HOX or PBX would be used to immunoprecipitate the chromatin. PCR amplification with primers within the *Hoxb1* ARE enhancer would follow to determine whether the acetylated or unacetylated proteins bind to the active ARE.

The ChIP assay is an exciting tool that can be used in the mouse embryo to study the kinetics of activation of *Hox* genes, for instance the activation of *Hoxb1* in r4 during development. Only in times of activation is the chromatin in an open configuration and the histones acetylated in the promoter of the activated gene. In these cases, one can use anti-acetyl lysine antibodies that recognize the acetylated histone tails to immunoprecipitate the chromatin (prepared for example from hindbrain cells of mouse embryos at different embryonic ages). PCR amplification with primers within the *Hox* gene of interest can be used to analyze the kinetics of activation of this *Hox* gene throughout developmental time. Similarly, one can study the timing of binding and

activation of specific transcriptional regulators, such as HOX, KROX20, KRML1,etc., on *Hox* gene promoters. For these studies, the antibodies need to be directed against the regulator proteins rather than against the acetylated histones.

How does acetylation of HOX and/or PBX affect their function in transcriptional regulation? One would expect acetylation, mediated by the coactivators CBP and P/CAF, to favor activation of transcription. CBP and P/CAF would not only acetylate the histone tails to alter chromatin structure but would also acetylate HOX and PBX to activate transcription. Acetylation of HOX and/or PBX may favor the formation of the HOX•PBX cooperative complex *in vivo* and enhance its binding to DNA. On the other hand, acetylation of HOX and/or PBX may lead to transcriptional repression. CBP and P/CAF would acetylate first the histone tails allowing transcriptional activation and subsequently acetylate HOX and/or PBX to terminate the activation. Acetylation by CBP and/or P/CAF may lead, for example, to dissociation of the HOX•PBX cooperative complex from DNA and hence repression of transcription. These possibilities await to be tested. Determination of which lysine is acetylated in HOX and PBX using site-directed mutagenesis of the lysines into arginines and *in vitro* acetylation of the mutant proteins would provide a preliminary insight into which of the two models is valid. Acetylation of the full-length proteins *in vitro* and EMSA analysis using the acetylated HOX and PBX, and a probe that accommodates the formation of the cooperative complex, would provide evidence of increased or decreased DNA-binding by the cooperative complex as a result of acetylation. This would also suggest whether acetylation would dissociate or stabilize the complex *in vivo*, favoring transcriptional repression *versus* activation. One should not exclude, however, that acetylation may have a dual function in regulating the

transcriptional activity of HOX•PBX. The involvement of two HATs in the acetylation of PBX may prove important in such a dual function. Each HAT may acetylate a different lysine in the PBX protein that may differentially alter its properties, with respect to interaction with HOX or binding to DNA, and its functions in transcriptional regulation. The results of the above proposed experiments would greatly contribute to our understanding of the transcriptional regulation by the HOX•PBX complexes and their functions during development and cancer.

APPENDIX

Figure 1: Targeting construct for the generation of an *in vivo* deletion in PBX1 (PBX1 Δ1-89-HA).

A knock-out/knock-in strategy would be used with this targeting construct to replace the wild-type *Pbx1* gene by a 1.1 kb cDNA encoding PBX1 Δ1-89-HA. The cDNA is cloned at the *Nsi*I site of the first exon in the *Pbx1* gene. This puts the cDNA at codon 9 of the first exon in frame with the *Pbx1* translation initiation codon. The PGK-neomycin gene flanked by two loxP sites, is cloned 3' to the PBX1 Δ1-89-HA cDNA. The 5' recombination arm is 3.3 kb and the 3' recombination arm is 2.7 Kb. The two probes to be used in the diagnostic Southern analysis were cloned together as *Pst*I fragments in the pKS vector (pKS-Probes). For Southern analysis, genomic DNA from mouse tails is to be digested with *Kpn*I, resolved on agarose gels, transferred to nitrocellulose membranes and probed with the diagnostic probes. The 5' probe (5'P) is 700 bp in length and would detect a 6.6 kb *Kpn*I fragment from the targeted allele, and the 3' probe (3'P) is 400 bp and would detect a 5.2 kb *Kpn*I fragment from the targeted allele. For wild-type alleles, both probes would detect an 8.5 kb *Kpn*I fragment. To linearize the targeting construct, *Eco*RV or *Bss*HII digests could be used.

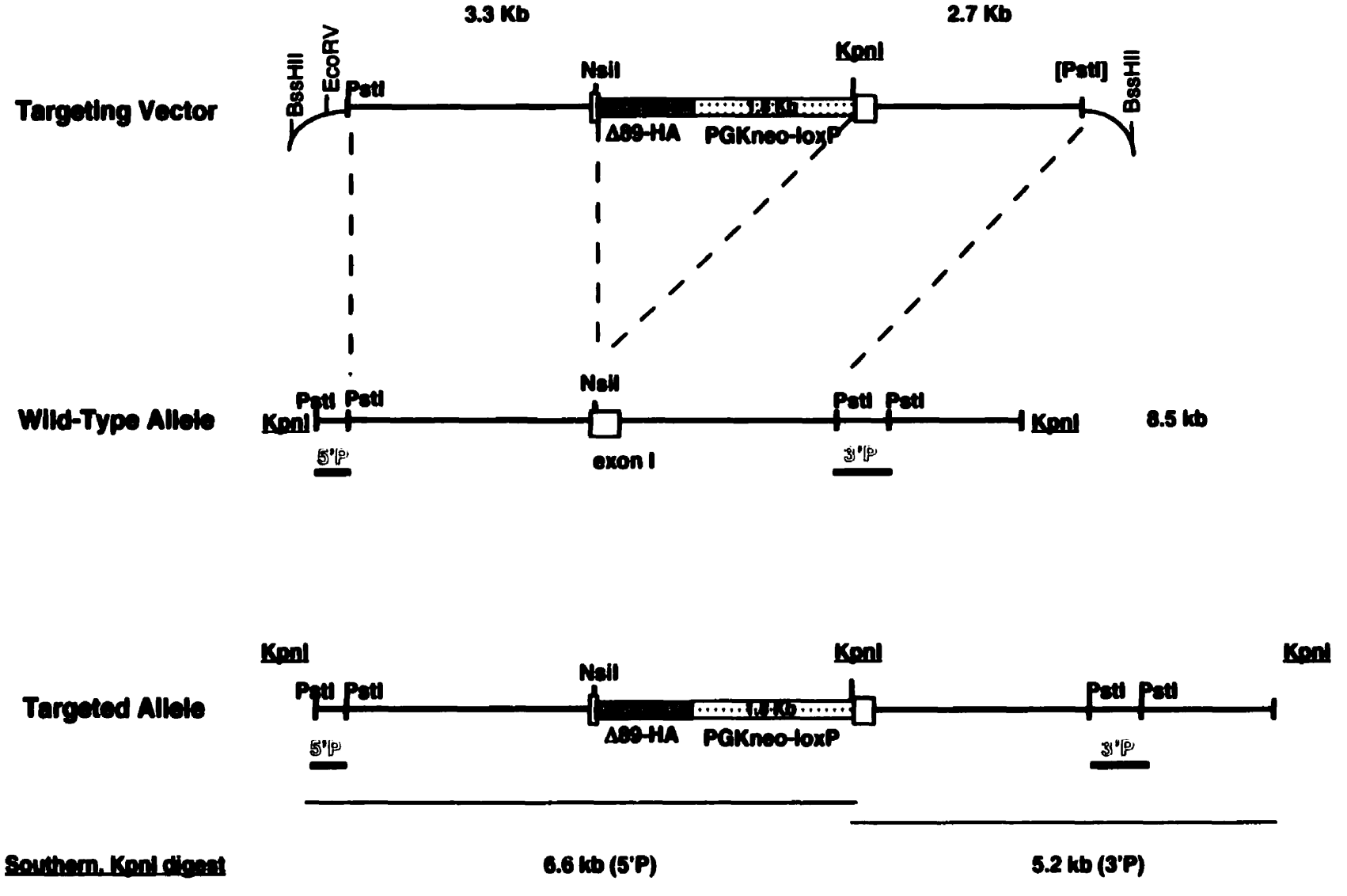

3.3 Kb
2.7 Kb
Targeting Vector
BssHII
EcoRV
PstI
NsiI
KpnI
[PstI]
BssHII
Δ89-HA
PGKneo-loxP
Wild-Type Allele
KpnI
PstI
PstI
NsiI
PstI
PstI
KpnI
8.5 kb
5'P
exon I
3'P
KpnI
KpnI
KpnI
Targeted Allele
PstI
PstI
NsiI
PstI
PstI
Δ89-HA
PGKneo-loxP
5'P
3'P
Southern, KpnI digest
6.6 kb (5'P)
5.2 kb (3'P)

Figure 2: Differential acetylation of HOXD4 and PBX by P/CAF and CBP.

(**A**) *In vitro* acetylation assay: 0.5 μg of recombinant HOXD4 or PBX HD were incubated with 200 ng of either P/CAF (P) or CBP (C) HAT proteins and ^{14}C-acetyl-coA at 30°C for 30 minutes. Labelled proteins were resolved on SDS-PAGE and detected by autoradiography.

(**B**) *In vivo* acetylation assay: HeLa cells were transfected with plasmids for either flag-HOXD4 or PBX along with those for flag-P/CAF or HA-CBP and were pulse-labeled with ^{3}H-acetate for 3 hours in the presence of 2 μM TSA. Immunoprecipitation reactions (IP) followed to detect *in vivo* acetylation of HOXD4 or PBX using M2 beads (α-flag) in the case of flag-HOXD4 or α-PBX antibodies in the case of PBX1A. IP products were resolved by SDS-PAGE and labeled proteins were detected by autoradiography.

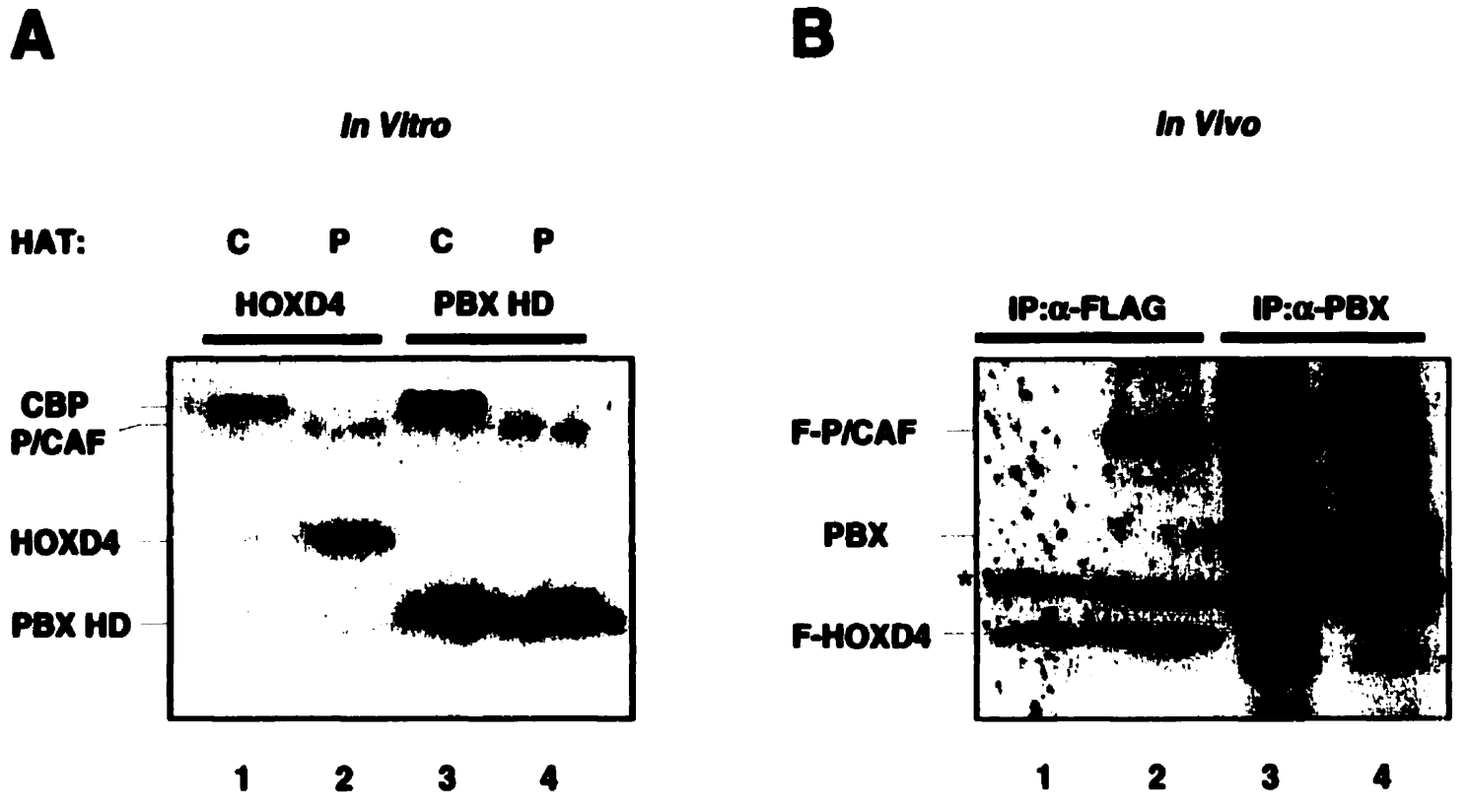
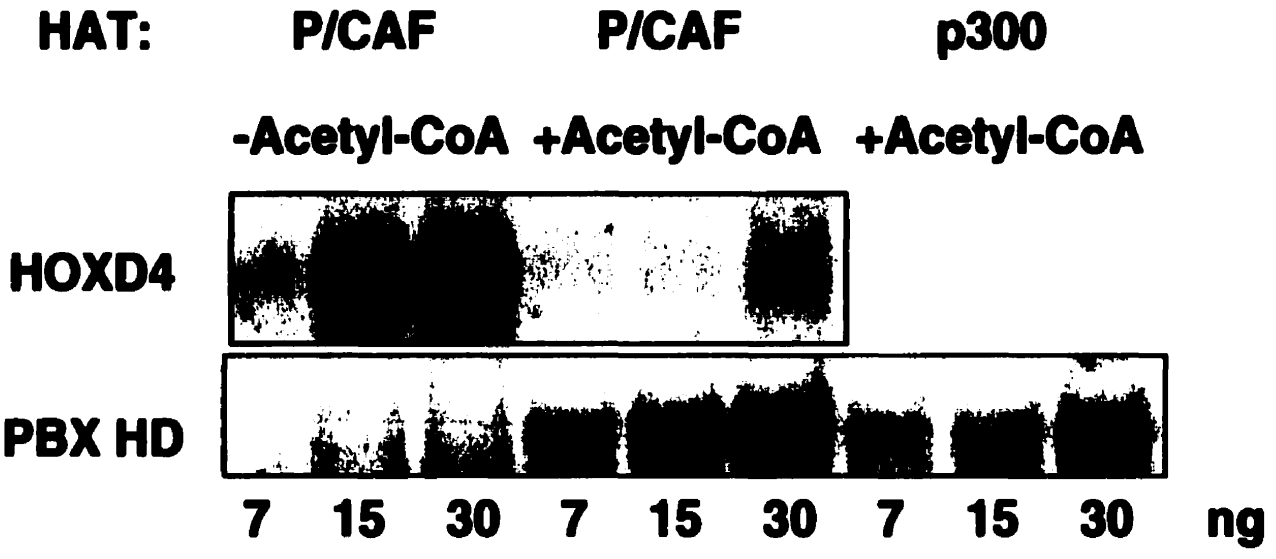

A
In Vitro
HAT:
C
P
C
P
HOXD4
PBX HD
CBP
P/CAF
HOXD4
PBX HD
1
2
3
4
B
In Vivo
IP:α-FLAG
IP:α-PBX
F-P/CAF
PBX
F-HOXD4
1
2
3
4

Figure 3: Opposite effects of acetylation on the DNA-binding abilities of HOXD4 and PBX HD.

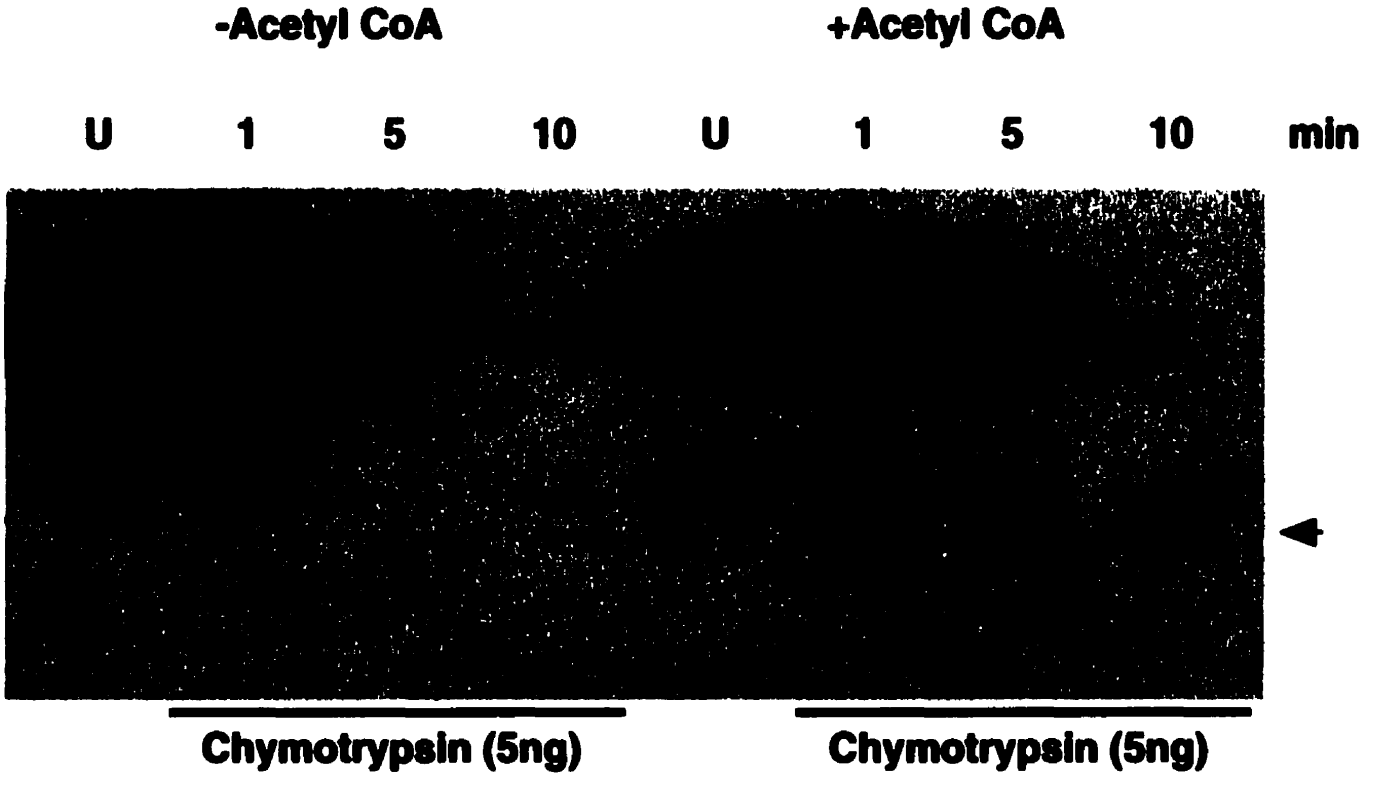
HOXD4 and PBX HD were acetylated *in vitro* as described in Fig. 2A. HOXD4 was acetylated with P/CAF while PBX was acetylated with either P/CAF or p300. Increasing amounts of the acetylated (+acetyl coA) or control unacetylated (-acetyl coA) proteins were subjected to EMSA on an oligonucleotide probe (P) bearing the HOX•PBX binding. Acetylation leads to inhibition of HOXD4 monomer binding while it improves that of PBX HD.



P : 5' TCACCATGATTGATGGGCGACTGCTCGG 3'

Figure 4: Conformational change of HOXD4 by acetylation with P/CAF.

HOXD4 was acetylated *in vitro* as described in Fig. 2A. Unacetylated control (-acetyl coA) or acetylated HOXD4 (+acetyl coA) were subjected to partial proteolysis with 5 ng chymotrypsin at 25°C for different time points. U, uncut. Acetylation induces a different digestion pattern as compared to that of the unacetylated protein, and to the protection of one digestion product (arrow), suggesting a change in conformation.


-Acetyl CoA
+Acetyl CoA
U
1
5
10
U
1
5
10
min
Chymotrypsin (5ng)
Chymotrypsin (5ng)

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