INTERPLAY BETWEEN CHROMATIN AND TRANS-ACTING FACTORS REGULATING THE Hoxd4 PROMOTER DURING NEURAL DIFFERENTIATION

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Running title: Chromatin and trans-acting factors regulate Hoxd4 transcription
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Correct patterning of the antero-posterior (AP) axis of the embryonic trunk is dependent on spatiotemporally restricted Hox gene expression. In this study, we identified components of the Hoxd4 P1 promoter directing expression in neurally differentiating retinoic acid (RA)-treated P19 cells. We mapped three nucleosomes that are subsequently remodeled into an open chromatin state upon RA-induced Hoxd4 transcription. These nucleosomes spanned the Hoxd4 transcriptional start site in addition to a GC-rich positive regulatory element located 3' to the initiation site. We further identified two major cis-acting regulatory elements. An autoregulatory element (ARE) was shown to recruit HOXD4 and its cofactor PBX1, and to positively regulate Hoxd4 expression in differentiating P19 cells. Conversely, the Polycomb Group (PcG) protein Ying-Yang 1 (YY1) binds to an internucleosomal linker and represses Hoxd4 transcription before and during transcriptional activation. Sequential chromatin immunoprecipitation (ChIP) studies revealed that the PcG protein MEL18 was co-recruited with YY1 only in undifferentiated P19 cells, suggesting a role for MEL18 in silencing Hoxd4 transcription in undifferentiated P19 cells. This study links for the first time local chromatin remodeling events that take place during transcriptional activation with the dynamics of transcription factor association and DNA accessibility at a Hox regulatory region.

Hox gene transcriptional activation marks the onset of an intricate series of events leading to proper embryonic patterning in all animals. The products of Hox genes, homeodomain-containing HOX transcription factors, are essential in specifying antero-posterior positional identity, hindbrain development, limb formation and numerous additional morphogenetic and organogenetic events (1,2). Given their crucial role in embryonic development, the genes encoding HOX proteins are highly conserved throughout the animal kingdom, and their expression is tightly regulated (3). In mammals, thirty-nine Hox genes are organized into four clusters, each located on a different chromosome (1). Comparison of the clusters reveals thirteen possible gene positions, although none of the clusters retains a full complement of thirteen genes. Hox genes occupying the same positions are termed paralogs, sharing high sequence identity and functional redundancy. One can assign a 3' and a 5' end to a cluster since all genes are transcribed in the same direction. A unique feature of Hox gene clusters is a process termed “colinearity,” correlating both the timing of transcriptional activation and the anterior expression borders with the position of a particular Hox gene along a cluster (4). Therefore, genes located more 3’ are expressed earlier and have a more anterior expression border than genes located more 5’ along the cluster. This observation and several other studies have led to the hypothesis that a sequential opening of chromatin, starting at the 3’ end of a cluster and moving successively 5’, leads to the release of silencing first at the 3’ end, and sequentially allowing the
expression of more 5' genes with increasing time (5,6). Numerous studies have now established that it is the strictly defined anterior expression border that most determines HOX activity, and shifting this border either anteriorly or posteriorly leads to embryonic malformations and homeotic transformations (7). A full understanding of Hox function therefore requires an explication of the mechanisms governing spatio-temporally restricted expression.

The regulation of Hox gene transcription is accomplished through a set of enhancers located a few kilobases upstream or downstream of the gene, although some enhancers have been shown to be located hundreds of kilobases away (8). Transcription factors involved in Hox gene regulation include HOX proteins themselves, acting with PBX, MEIS and PREP cofactors, (9,10), Cdx family proteins(11), AP-2 (12), Kreisler (13), KROX20 (14), and Sox-Oct family members(15). One of the most important factors regulating Hox gene expression is RA (16).

Functional retinoic acid response elements (RAREs) have been identified for Hoxa1, Hoxb1, Hoxa4, Hoxb4, Hoxd4, Hoxb5, Hoxb6, and Hoxb8 (17). In vivo mutations of the Hoxa1 and Hoxb1 RAREs result in hindbrain patterning defects and cranial nerve malformations similar to those observed in the Hoxa1 and Hoxb1 full knockouts, emphasizing a key role for retinoids in controlling Hox gene expression during embryogenesis (17).

Transgenic studies in mouse embryos where RARE sequences were mutated resulted in posteriorized Hox gene expression in both the somitic mesoderm and the developing hindbrain (18,19). This, in addition to tissue culture studies (20), suggests a positive role for RA in activating Hox gene transcription.

Hoxd4 is an ortholog of the Drosophila Hox gene Deformed (Dfd). Hoxd4 expression begins at embryonic day (E) 8.25 and has an anterior border of expression between rhombomeres six and seven (r6/7) in the developing hindbrain, and between somites four and five in the mesoderm (21). A 5' mesodermal enhancer containing an RARE, and an ARE, have been described earlier and shown to be functional in P19 cells (10,20). A 3' neural enhancer containing a DR5 type RARE is crucial for initiation and maintenance of Hoxd4 expression in the CNS (22-24). Two proximal promoters, an upstream promoter (P2) and a downstream promoter (P1) have been identified (25). Transcripts originating from P1 have a more anterior border of expression in the CNS (r6/7), and are further anteriorized in response to RA, suggesting that P1 is more responsive to signals originating from the 3' neural enhancer (25).

Similar to Hoxd4 (26), interactions between the Hoxd4 3' neural enhancer and its proximal promoter P1 are important in initiation of Hoxd4 gene expression in the hindbrain of transgenic embryos (19), and in neurally differentiating P19 embryonal carcinoma cells (27). We have correlated this enhancer-promoter interaction with chromatin changes occurring upon Hoxd4 gene activation in response to RA in neurally differentiating P19 cells, and in the central nervous system (CNS) of developing mouse embryos. Chromatin opening occurred first at the 3' neural enhancer, followed by the intervening sequences, culminating at the proximal promoter P1 (27). These studies also established P19 cells as a valid system for studying Hoxd4 enhancer-promoter function.

In this study, we further characterized the Hoxd4 P1 promoter. Because of the importance of enhancer-promoter communication and chromatin modifications that culminate at P1 during neural Hoxd4 gene expression, we investigated the roles of nucleosome position, chromatin remodeling and cis-regulatory elements involved in initiating Hoxd4 gene expression in neurally differentiating P19 cells. We show that nucleosomes are positioned at P1 and are remodeled in response to RA. Furthermore, we show that an ARE and a YY1 binding site regulate correct Hoxd4 expression in P19 cells in response to RA, as does a GC-rich motif that is essential for core promoter activity. We also show that YY1 continues to exert a repressive effect on Hoxd4 transcription in P19 cells even after gene activation. This repression is correlated with MEL-18 recruitment to the YY1 binding site at P1 during Hoxd4 transcriptional silencing. Finally, we discuss our results in the light of the evidence that links chromatin to Hox gene regulation, and YY1-mediated repression to PcG-mediated silencing of Hoxd4.

Materials and Methods

Tissue culture and transfections - P19 mouse
embryonal carcinoma (EC) cells were cultured in alpha minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum. For neural differentiation, P19 cells were plated as low-density monolayers at 10^5 cells/ml of α-MEM supplemented with 0.3 μM retinoic acid (RA) for 48 h. Transient transfection was performed using Lipfectamine 2000 reagent (Invitrogen). P19 cells were plated either in the presence or absence of RA followed by transfection of different constructs in an antibiotic free α-MEM medium. Twenty-four hours later, the medium was replaced with α-MEM containing antibiotics, and RA was added to the cells undergoing differentiation. Transfected cells were harvested 24 hours later by scraping in ice-cold phosphate buffer saline (PBS) and resuspended in 100 µl lysis buffer (10% Triton X-100, 1 M K_2HPO_4, 1 M KH_2PO_4, 1 M DTT) for 5 min.

Luciferase vectors and assays - Luciferase reporter constructs were designed in a pXP2 promoter-less background (28) containing the luciferase gene coupled to region CL of the Hoxd4 3’ neural enhancer (pXP2CL, construct 1, Fig. 2B) (24). pXP2CL was prepared by cloning Eco RV/Bam HI-digested CL fragments (in TOPO-II background) into Sma I/Bam HI digested pXP2 followed by sequencing. All Hoxd4 P1 deletion fragments were amplified by PCR using P1-specific primers and Pfx platinum (Invitrogen) as the heat-stable DNA polymerase. pSNlacZpA (22) was used as a template, and PCR products were subcloned into TOPO-II (Invitrogen) and sequenced. Bam HI-Xho I promoter fragments were cloned into Bgl II/Xho I sequentially digested pXP2CL. For luciferase assays, 30 µl of cell extract was incubated in 100 µl luciferin solution (10 mM luciferin, 1 M Tris pH 7.8) and 12.5 µl assay buffer (50 mM ATP, 1 M MgCl_2, 1 M Tris pH 7.8). Luciferase activity was measured using a Lumat LB 9507 luminometer (EG&G Berthold). For measuring transfection efficiency, RSV β-gal plasmid was co-transfected with the luciferase reporter constructs and β-galactosidase assays were performed as described (27). Final luciferase values were reported as relative luciferase activity per β-gal unit.

site-directed mutagenesis - For site-directed mutagenesis of the YY1 binding site, primers carrying the mutated sequences (Table1) were used in two separate PCRs (each containing either the 5’ or 3’ primer containing the mutation), the amplified products were gel-purified, combined, and used as a template for another nested PCR. The products of the final PCR were also gel-purified, sub-cloned into TOPO-II and sequenced to verify mutations. This was followed by Bam HI/Xho I digestion and cloning into pXP2CL. For mutating the ARE sequences, an Nru I-Bam HI fragment was released from pSXm34/35 already harboring the mutated ARE (10) blunt ended and cloned into construct 1 (Fig. 2B).

Micrococcal nuclease (MNase) digestion, genomic DNA purification - Nuclei were prepared according to Carey and Smale (29). Undifferentiated or neurally differentiated P19 cells were resuspended in NP-40 lysis buffer for 5 min, pelleted and resuspended in MNase digestion buffer. This was followed by adding either 1 u or 5 u of MNase (Roche) for 5 minutes. The reaction was stopped and samples were treated with 25 µg/µl proteinase K (Roche) overnight at 37 °C. For naked DNA controls, MNase digestion was carried out following DNA purification of chromatin extracted from P19 cells. Following phenol/chloroform extraction, samples were treated with 10 µg/µl RNase A for 2 hours, followed by another round of phenol/chloroform extraction. Finally, DNA was ethanol-precipitated and resuspended in 100 µl H_2O. One µg DNA was used for ligation-mediated PCR (LM-PCR, see below).

Restriction Enzyme accessibility - Following nuclei purification, samples were resuspended in restriction enzyme digestion buffer (29) followed by incubation with different concentrations of Kpn I and Pvu II (NEB Biolabs) for either 10 or 20 min at 37°C. This was followed by proteinase K digestion and DNA purification. After determining DNA concentration, 1 µg DNA was used for in vitro Eco RI (NEB Biolabs) digestion. Twenty-five percent of the digestion reaction was used for LM-PCR.
Ligation-mediated PCR (LM-PCR) - LM-PCR was performed as previously described (30), with some modifications. Following DNA purification, digested DNA was ligated to a double-stranded unidirectional linker, which provided a common 5' sequence for annealing to a PCR primer. This was followed by PCR amplification using a 3' gene specific primer and a 5' primer complementary to the unidirectional linker (Table 1). Finally, a second nested PCR was performed using a radioactively labeled 3' gene specific primer, allowing us to detect specific MNase-cleavage products by autoradiography. Because the PCR templates consist of DNA fragments linked to a 25 bp linker, the actual size of the gene specific product is 25 bp less than the size detected on the gel. All PCR reactions were carried out with the heat-stable DNA polymerase Pfu (Fermentas). First-strand synthesis reactions were performed for restriction enzyme–treated DNA, but not for MNase-treated DNA. Instead, MNase-treated DNA was phosphorylated using polynucleotide kinase (PNK, Fermentas), followed directly by ligation with the unidirectional linker (Table 1). The amplification PCR consisted of 18 (restriction enzyme) or 23 (MNase) PCR cycles with an extension time starting at 5 min plus 15 s for each additional cycle. The labeling PCR consisted of three cycles for all the in vivo DNA treatments. Gene specific primers complementary to Hoxd4 P1 were used and their exact positions are given in Table 1. Real-time PCR using gapdh-specific oligos was performed to ensure equal loading.

ChIP assays and real-time PCR - ChIP experiments were performed as described (27). Oligonucleotides used for real-time PCR are listed in Table 1. For sequential ChIP, a second immunoprecipitation procedure was performed using chromatin samples consisting of antibody–immunoprecipitated DNA complexes that were eluted from the agarose beads of the first ChIP. Five µg of purified antibodies were used for each ChIP experiment. Anti-PBX1, -MEI18 and -YY1 antibodies were purchased from Santa Cruz Biotechnology. The anti-HOXD4 rabbit polyclonal antibody was described previously (Rastegar et al., 2004).

Electromobility Shift Assays (EMSAs) and supershifts - Nuclear extracts and EMSAs were performed as described (31). Five µg of polyclonal antibody were added to the binding reaction in supershift experiments. The primers used are listed in Table 1. Anti-Sp1 and anti-Sp3 antibodies were kind gifts of Dr. Christopher Mueller (Queen’s University).

Whole cell extracts and immunoblotting - Whole cell extracts from P19 cells and immunoblotting were performed as described (27).

RNA extraction and RT-PCR - RNA extraction and RT-PCR were performed as described (27). For PCR, primers specific for the Hoxd4 homeobox (located in the second coding exon) were used to assay for Hoxd4-specific transcripts, in addition to gapdh-specific primers (spanning the coding region) that were used as a control (Table 1).

RNA interference - For silencing yy1 gene expression, siRNA oligonucleotides based on yy1 cDNA sequences were used as previously described (32) (accession number NM 009537). The YY1 siRNA sequence is identical in the human, mouse, and Xenopus cDNAs. Control siRNA oligonucleotides were designed as purine to pyrimidine (and vice versa) mutations of the YY1 siRNA primers (Table 1). For YY1 knockdown in undifferentiated P19 cells, both control and YY1 siRNA primers having Bgl II and Hind III compatible ends were annealed and cloned into Bgl II/Hind III digested pSUPER (OligoEngine, Seattle, WA) creating pSUPER-ctrl and pSUPER-YY1, respectively. Plasmids were transfected into undifferentiated P19 cells using Lipofectamine 2000 and knockdown was achieved 24 hours later. For P19 cells differentiated with RA, the same cDNA sequences were used for designing the siRNA primers, this time delivered to the cells as ready to use 2’-deprotected double stranded siRNA duplex. The pSUPER-retro system was not used in these experiments because P19 cells had to be pre-treated with RA before transfection, optimal timing of YY1 knockdown did not coincide with initiation of Hoxd4 transcription, and therefore the effects of YY1 depletion on Hoxd4 gene expression could not be monitored. Instead, P19 cells were treated with
RA for 24 hours followed by transient transfection with YY1 siRNA oligonucleotides (YY1 siRNA) or control oligonucleotides (Ctrl siRNA). The cells were subjected to total protein and RNA extraction 24 hours later. Both primer sets were purchased from Dharmacon Research Inc., and transfected in P19 cells at a concentration of 200 nM using Lipofectamine 2000. Cells were harvested 48 h later.

RESULTS

Nucleosome positioning and remodeling at the Hoxd4 P1 promoter

In our previous study, we showed that Hoxd4 transcription is initiated from the correct start sites within 48 hours following RA treatment of P19 cells (27). RA responsiveness was dependent on the Hoxd4 3’ neural enhancer which contains an RARE. Moreover, modifications characteristic of transcriptionally active chromatin (histone H3 acetylation and methylation) were correlated with transcriptional activation, starting at the 3’ end of the gene and concluding more 5’ at P1. This conversion was accompanied by recruitment of RNA polymerase II (Pol II) to the Hoxd4 locus, a process that did not occur in the absence of RA, and not before those chromatin modifications took place. These results suggested that the Hoxd4 P1 promoter is not a nucleosome-free region, and raised the question of whether nucleosome positioning plays an active role in mediating the repression and/or activation of Hoxd4 transcription.

To investigate nucleosome positioning at P1, we performed high resolution nucleosome mapping using MNase digestion coupled with LMPCR analysis (45). A unique property of MNase is its ability to create double-stranded nicks in inter-nucleosomal regions of partially digested chromatin. To compare nucleosome positions before and after Hoxd4 transcriptional activation, we used nuclei extracted from P19 cells either untreated, or treated with RA for 48 hours and thus coinciding with initiation of Hoxd4 transcription. Two sets of nested primers were designed to confirm nucleosome positions at P1, one set amplifying in the 3’ direction relative to Hoxd4 transcription, and the other in the 5’ direction (Fig. 1A). Both primers were located upstream of the transcriptional start site (+1) at positions -115 (O-115) and -69 (O-69). Primer positions were chosen based on results from low resolution MNase-coupled southern blotting experiments that gave a rough estimation of nucleosome positions (data not shown). Two major cleavage products, could be detected using radiolabeled O-115 and nuclei from untreated cells, but not the naked DNA control (Fig. 1B, left panel). These sites thus define an inter-nucleosomal region spanning nucleotides -25 to -10 respectively. This indicated that a nucleosome (N2) is positioned at P1 starting at -25 and extending more 5’. Moreover, the lack of significant cleavage products 3’ to nucleotide -10 suggested that an additional nucleosome (N3) is positioned at P1 with a 5’ border at position -10 (Fig. 1B, left panel).

We then compared cleavage products of RA-treated versus untreated nuclei. There was a significant increase in intensity of PCR products in RA-treated samples following cleavage at two different MNase concentrations (1u and 5u) (Fig. 1B, left panel). However, the position of the two major cleavage products was unchanged, indicating that nucleosome sliding did not take place following RA treatment (fig. 1B, left panel), suggesting a more relaxed state of chromatin, making DNA more susceptible to enzymatic digestion.

To confirm the position of N2, we designed an additional set of PCR primers for amplification in the 5’ direction (Fig. 1A). Two major cleavage products were detected with O-69 corresponding to gene specific products starting at position -69 and extending till positions -171 and -183, respectively (Fig. 1B, right panel). These products were not detected using naked DNA. Importantly, these results confirm the position of N2 obtained with O-115 (Fig. 1A), fixing a nucleosome unit length of 146 bp with borders at positions -25 and -171. This also suggests that a third nucleosome (N1) is positioned upstream of N2 with a 3’ border at position -183. PCR products from samples treated with RA were significantly more intense compared to untreated samples suggesting that the latter were protected by the presence of more compacted chromatin. To ensure that equal amounts of starting material were used, we performed real-time PCR using gapdh-specific oligos which indicated that comparable DNA levels were present in the different samples (Fig. 1C). Therefore, these
results suggest that upon RA treatment, chromatin relaxes at P1, is not accompanied by nucleosome sliding, and leads to transcriptional initiation at Hoxd4 P1. We note that the relative paucity of MNase I cut sites in naked DNA may suggest that our approach has not definitively proven that nucleosomes are positioned at the Hoxd4 P1 promoter. However, several major sensitive sites on naked DNA are indeed masked in chromatin preparations, and the unit nucleosome distance of 146 bp defining the span of N2 is unlikely to occur by chance.

To further investigate chromatin remodeling at P1, we performed restriction enzyme accessibility assays focusing on N2 (Fig. 1D). To do this, Kpn I and Pvu II digests were carried out using chromatin extracted from P19 cells either untreated or treated with RA for 48 hours, followed by LMPCR using the gene specific primers (O-115 and O-195 coupled with Kpn I and Pvu II digests, respectively). Both Kpn I and Pvu II restriction sites were hypersensitive to increasing concentrations of enzyme in samples treated with RA as revealed by the increased intensity of cleavage products (Fig. 1D, compare Kpn I lanes 1 and 2, 3 and 4, 5 and 6; Pvu II lanes 7 and 8, 9 and 10, 11 and 12). These results show that chromatin remodeling at P1 coincides with transcriptional initiation, corroborating the role of chromatin decondensation in activating Hoxd4 gene expression.

**Mapping of cis-acting regulatory elements at P1**

Nucleosome positioning and modification are coordinated with the placement of cis-regulatory elements to control transcriptional status. To determine the DNA sequences necessary for correct Hoxd4 expression from P1 relative to nucleosome positioning, we constructed luciferase reporters driven by the Hoxd4 P1 promoter (-800 to +140) and containing 540 bp of the RA-responsive Hoxd4 neural enhancer region (pXP2CL-Hoxd4P1, construct 3) (Fig. 2A,B). 5′ to 3′ sequential deletion constructs were used in transient transfection assays using both undifferentiating and neurally differentiating P19 cells (treated with RA for 48 hours). Control experiments showed that neither the 3′ neural enhancer nor P1 promoter sequences alone could direct significant reporter activity (Fig. 2B, constructs 1 and 2). Therefore transcriptional initiation can only be attributed to the action of the neural enhancer on the P1 promoter.

Maximum RA-responsiveness was achieved using construct 3 containing the full 940 bp of P1 sequences. Sequential deletion of P1 sequences identified two key regulatory units. P1 region -800 to -580 possessed strong activator function, since deletion of these sequences decreased reporter gene expression by ten-fold (construct 4, Fig. 2B). A second regulatory element spanned P1 at positions -195 to -115. Deletion of these sequences resulted in increased reporter gene expression, suggesting the presence of significant repressor elements (Fig. 2B, constructs 5 and 6). Further deletions of all P1 sequences upstream of the transcriptional start site (+1) did not impair promoter activity (Fig. 2B, construct 7). Deletion of sequences +66 to +140 only slightly decreased reporter activity indicating that crucial proximal promoter elements lie between +1 to +66 (Fig. 2B, construct 8). The presence of 3′ promoter elements has been shown for several proximal promoters including Hoxb4, Hoxa4 and TAF65 (33).

The Hoxd4 ARE is a positive regulator of P1

The positive regulatory region between –800 and –580 has previously been shown to harbor an ARE (Fig. 3A) (10). Two TAAT/ATTA motifs that bind HOXD4 in vitro are functional components of the ARE. Mutation of both sequences results in decreased transcriptional activity of reporter constructs driven by promoters P1 and P2 (10). However, those studies were conducted in undifferentiated P19 cells together with co-transfected HOXD4 expression vectors, and therefore did not address the direct role of the ARE on enhancer-dependent transcription at P1. While HOXD4 antibodies were able to supershift complexes formed between nuclear extracts and primers containing the ARE sequence in EMSA (10) a direct role of the Hoxd4 ARE in vivo was not addressed. To address the role of the ARE in regulating transcriptional initiation at Hoxd4 P1, both TAAT/ATTA motifs of the ARE were mutated simultaneously in construct 3. (mARE). Mutating both AREs dramatically decreased reporter activity in the presence of RA (Fig. 3B), supporting a key role for the ARE in regulating transcriptional initiation from P1.

To assess HOXD4 binding to the ARE in
vivo, we performed ChIP experiments on the endogenous *Hoxd4* locus using an anti-HOXD4 antibody and primers spanning the ARE. There was significant recruitment of HOXD4 to the *Hoxd4* ARE but not to the gapdh control locus following RA treatment (Fig. 3C).

The *Hoxd4* ARE does not harbor typical sites for cooperative binding of HOXD4 and PBX. However, Extradenticle (EXD), the *Drosophila* ortholog of PBX1, has been shown to modify DFD binding to the *Dfd* ARE despite a similar absence of EXD binding sites (34). We therefore tested whether PBX1 is bound to the *Hoxd4* ARE in P19 cells by performing ChIP experiments using anti-PBX1 antibodies. Our results showed that PBX1 was significantly and specifically recruited to the ARE following RA treatment (Fig. 3D), implying possible tethering of PBX to the ARE via protein-protein interactions with HOXD4. ChIP experiments using antibodies against another HOX partner, MEIS1, revealed no significant binding (Fig. 3E).

**YY1 represses transcription from P1**

A negative regulatory element was mapped between positions -195 and -115 (Fig. 2B). We scanned this sequence for possible known transcription factor consensus binding sites. A CCAT core plus flanking sequences at position –182, located precisely in the short linker separating nucleosomes N1 and N2, bore high similarity to the consensus binding site for transcription factor YY1 (Fig. 4A). YY1 was of special interest given its role in *Hoxb4* gene expression, with binding sites at both the *Hoxb4* promoter and intronic enhancer. To investigate whether YY1 binds *Hoxd4* P1 at this region, we performed EMSAs using P1 probes spanning the CCAT-containing sequence (d4-YY1) (Fig. 4B), and nuclear extracts obtained from P19 cells either untreated or treated with RA for 48 hours. Although the expression level of YY1 does not change following RA treatment (data not shown), we used extracts from RA-treated and untreated P19 cells to monitor changes in protein modification or protein-protein interactions that might influence DNA binding. As shown in Figure 4B, a specific protein-DNA complex could be detected using the d4-YY1 probe, and complex formation was specifically inhibited either by cold probe or a YY1 consensus site. A nonspecific competitor did not inhibit complex formation (Fig. 4B). The presence of YY1 in the shifted complex was assessed with anti YY1 antibodies. As shown in Figure 4C, the protein-DNA complex was significantly inhibited by anti YY1 antibodies, but not with several other nonspecific antibodies (Fig. 4C), further supporting the presence of YY1 in the shifted complex.

The relevance of YY1 to P1 function was investigated by mutation of its binding site in Construct 4 (Fig. 5A, mYY1). Mutating the YY1 site led to an increase in reporter gene expression (Fig. 5B) that was comparable to that seen following deletion of sequences -195 to -115 (Fig. 2B, constructs 5 and 6). To assess whether this activity could be correlated with the presence of YY1 at this site in vivo, we performed ChIP experiments using chromatin extracts from RA-treated and untreated P19 cells, and immunoprecipitating with anti YY1 antibodies. Significant levels of YY1 were present at the endogenous P1 YY1 binding site in neurally differentiating RA-treated P19 cells as well as in untreated cells (Fig. 5C), indicating that YY1 binds to P1 before and after transcriptional activation. This observation suggests a role in modulating *Hoxd4* expression even after transcription has been initiated.

We reasoned that YY1 may maintain *Hoxd4* in a repressed state in undifferentiated P19 cells. Following neural differentiation, YY1 could either switch to an activator, as it does at the *IFNB* gene (35), or could persist as a repressor, thereby balancing stimulatory signals to achieve appropriate amounts of *Hoxd4* transcripts. To examine the functional consequences of YY1 binding, we used siRNA to knockdown *yy1* expression. P19 cells were transiently transfected with *yy1* siRNA and control expression vectors, and total protein and RNA were extracted 48 hours later. There was a significant decrease in YY1 protein expression following *yy1* knockdown in two independent experiments (Fig 6A, upper panel), which was not the case in untransfected cells or cells transfected with the control vector. Actin levels were equivalent in all samples. *Hoxd4* transcript levels were determined by RT-PCR (Fig. 6A, lower panel). *yy1* knockdown, but not control transfections, markedly stimulated endogenous *Hoxd4* expression (Fig. 6A, lower panel). These results were reproducible and
specific for Hoxd4, as gapdh levels remain unchanged. These data strongly suggest that YY1 represses Hoxd4 transcription, and that even partial loss of YY1 relieves this silencing despite the absence of RA.

To investigate whether YY1 acts as an activator or a repressor following Hoxd4 induction, we performed YY1 knockdown by direct transfection of siRNA double-stranded oligonucleotides (32). As shown in Figure 6B, YY1 levels were significantly reduced following transfection with YY1 siRNA oligonucleotides while they remained constant in untransfected cells and cells transfected with control siRNA oligonucleotides. Actin levels remained the same in all samples tested, confirming that YY1 knockdown was specific. Interestingly, RT-PCR results showed that following YY1 knockdown Hoxd4-specific transcript levels were significantly increased in both experiments, while gapdh RNA levels remained constant, suggesting that YY1 continues to repress Hoxd4 expression even after Hoxd4 induction.

One mechanism of YY1-mediated transcriptional repression is through recruitment of PcG proteins (36,37). In situ hybridization experiments have shown that Hoxd4 transcription is affected in mouse embryos null for the PcG gene Mel18 (38,39). We speculated that repression of Hoxd4 by YY1 may be mediated via a Mel18-dependent mechanism. We therefore conducted ChIP experiments to assess Mel18 recruitment to P1 before and after Hoxd4 transcriptional activation. Mel18 was indeed significantly recruited to Hoxd4 P1 in undifferentiated P19 cells, but was lost following RA treatment (Fig. 6C). To confirm that this loss was not due to a global decrease in Mel18 protein expression or to its relocalization to the cytoplasm, we separated nuclear and cytoplasmic fractions from both monolayer and RA-treated P19 cells. This was followed by immunoblot analysis using Mel18 specific antibodies (Fig. 6E). Expression of Mel18 was evident in both nuclear and cytoplasmic fractions, and was not significantly altered in either extract following RA treatment (Fig. 6E). TFIID was almost entirely localized to the nuclear fractions, indicating efficient separation of nuclear and cytoplasmic components (Fig. 6E). Hoxd4 expression was detectable only in RA-treated cells as confirmed by immunoblots using HOXD4 specific antibodies (Fig. 6E). These results confirm that the reduced association of Mel18 at P1 in P19 cells following RA treatment is indeed due to decreased recruitment of Mel18 to the Hoxd4 locus, as opposed to a global decrease in protein expression or subcellular relocalization.

To determine whether YY1 and Mel18 are present together at P1, extracts immunoprecipitated with α-YY1 antibodies were subjected to a second round of ChIP using Mel18 antibodies. As shown in Figure 6D, YY1 and Mel18 were bound together to P1 only in undifferentiated P19 cells, (Fig. 6D) suggesting that Mel18 is released from a P1-bound YY1 complex following RA treatment and Hoxd4 transcriptional activation. This further implies that the repressive effects of YY1 following Hoxd4 induction must be mediated through a Mel18-independent mechanism.

A positive regulatory element resides downstream of the transcriptional start site.

Finally, we attempted to identify the regulatory element responsible for core promoter activity located between positions +1 to +66. Two Luc reporter constructs were designed carrying sequences +1 to +33 (construct 8) or +34 to +66 (construct 9) and their activities compared to construct #6 (Fig. 2B) in RA-treated P19 cells. While construct 9 had comparable reporter activity to construct 6, that of construct 8 was significantly diminished (Fig. 7A). Consistent with activity mediated by the downstream region, oligonucleotides spanning 1-66 and 34-66 formed a single major complex with nuclear extracts in EMSA, contrary to primer 1-33 which showed no significant complex formation (Fig. 7B). Specificity of complex formation was then tested by EMSA using primer 34-66 as a probe accompanied by competition reactions using increased amount of cold probe (Fig. 7C). Interestingly, the specific binding of primer 34-66 to P19 nuclear extracts was accompanied by increased binding to extracts from RA-treated P19 cells (compare – to +). Sequences +34 to +66 contain a GC-rich sequence comparable to an Sp1/Sp3 consensus binding site. Given that an increase in complex formation was observed when nuclear extracts from RA-treated P19 cells were used, and that Sp1 DNA-binding ability is
increased in the presence of RA (40,41), we performed supershift experiments to investigate Sp1 and Sp3 as possible binding candidates. However, incubation with either Sp1 or Sp3 antibodies did not inhibit or supershift complex formation (data not shown), suggesting that a transcription factor other than Sp1 or Sp3 binds this region.

**DISCUSSION**

The role of chromatin in regulating Hox gene transcriptional initiation has recently come under the spotlight through studies showing sequential 3’ to 5’ chromatin decondensation of the HoxB cluster during mouse embryonic development (5,42), and regulation of HoxD gene expression through chromatin remodeling during limb development (43) and neural differentiation (27). In this study, we showed that nucleosomes are positioned at the Hoxd4 P1 promoter, and are remodeled following RA treatment of P19 cells. This remodeling leads to chromatin decondensation and coincides with the initiation of Hoxd4 gene transcription. We also identified three major cis-acting regulatory elements impinging upon P1: an ARE and a GC-rich element acting as positive regulators, and a YY1-binding site that represses P1 transcription before and during neural differentiation in P19 cells.

Chromatin remodeling can result in transcriptional repositioning of a nucleosome, such as is achieved by nucleosome sliding, and subsequent exposure of a transcription factor binding site, as is the case for the IFN-β promoter (44). For promoters like IL-12 p40, however, covalent histone modifications may lead to chromatin decondensation without altering nucleosome position (45). In this study, we showed that three nucleosomes are positioned at P1 in undifferentiated P19 cells where Hoxd4 transcription is repressed. N3 was shown to span the transcriptional start site (+1) and the positive GC-rich cis-element (+34 to +66) implying a functional relevance for N3 positioning. We have already shown that histone H3 is not acetylated at lysine 9 in the region of P1 in undifferentiated P19 cells, confirming a state of closed transcriptionally inactive chromatin (27). Upon RA treatment and P19 cell differentiation down the neural pathway, chromatin relaxation takes place at P1 as shown by MNase- and restriction enzyme-coupled LM-PCR experiments (Fig.1), but is not accompanied by nucleosome repositioning. This chromatin relaxation might be a direct consequence of the CBP recruitment and histone hyperacetylation known to take place during Hoxd4 transcriptional activation (27).

SWI/SNF and ISWI are two major ATP-dependent chromatin remodeling complexes. Whereas SWI/SNF is able to increase nucleosomal accessibility to DNase and restriction enzymes in the absence of translational movement, ISWI cannot. This feature of SWI/SNF-mediated remodeling has been proposed to result from changes to DNA topology, the conformation of the histone octamer or both. In one scenario, the energy of ATP would be used by SWI/SNF to generate a transient topological intermediate that would collapse into a stable and conformationally altered state (46). The differential ability of SWI/SNF to induce such chromatin changes in the absence of translational movement has been ascribed to the ATPase domain of the SWI-SNF-specific motor protein BRG1 (47). Together, these results suggest that the Hoxd4 promoter is a target of SWI/SNF, but not ISWI.

Maintenance of a repressed or an activated Hox transcriptional status has long been attributed to members of the PcG and Trithorax protein families (48,49) which execute their functions through chromatin modification. In this study, we describe a repressive role for YY1 in regulating Hoxd4 expression. YY1, a homologue of *Drosophila* PcG group protein Pleiohomeotic (PHO), is a multifunctional zinc-finger containing transcription factor that acts either as a transcriptional activator or repressor (50). YY1 mutant embryos die shortly following implantation and heterozygous embryos develop neurulation defects (51). Loss of function studies performed in *Xenopus* report antero-posterior patterning defects, reduced head structures, and abnormalities in midbrain-hindbrain boundaries consistent with a role for YY1 in CNS development and the control of Hox gene expression. Interestingly, YY1 has been shown to activate Hoxb7 gene expression in tissue culture (52), and to bind Hoxb4 promoter and CR1 enhancer region as part of an overlapping NFY/YY1 site (53). This site seems to be conserved for Hoxb4 and might also be present in the Hoxc8 early enhancer (53). However, the YY1...
binding site described in this study does not resemble the *Hoxb4* binding site since it does not seem to contain an overlapping NFY binding motif, and is not evolutionarily conserved with zebrafish, unlike other more downstream P1 sequences (19), suggesting a novel role for YY1 in fine tuning *Hoxd4* expression levels in mammalian embryonic development.

YY1 binds specifically to the -183 site *in vitro*, and to a short region spanning this site *in vivo*, and mutation of this sequence in reporter constructs relieves the repressive effect on transcriptional initiation from P1. Interestingly, this YY1 binding site is located within the internucleosomal region separating N1 and N2. YY1 binding to this site might not be significantly altered following nucleosome remodeling at P1 after RA treatment, since nucleosome sliding does not take place. Alternatively, prior YY1 binding may impede both nucleosome positioning and a subsequent closed chromatin state. Regardless, our results show that this site is accessible to YY1 even in the repressed state where positioned nucleosomes are condensed and *Hoxd4* is not transcribed.

Our RNA interference results indicate that YY1 represses *Hoxd4* gene expression in undifferentiated P19 cells where *Hoxd4* is known to be silenced (27). The fly homolog of YY1, PHO, binds Polycomb response elements (PREs) and recruits two types of PcG complexes, one of which contains the fly homolog of MEL18, Posterior sex combs (PSC) (54). Recently, data from Srinivasan *et al.* demonstrate that YY1 binds to PREs in *Drosophila* and subsequently recruits other PcG proteins to DNA (36). Similarly, our ChIP results are consistent with recruitment of MEL18 to the P1 YY1 site in undifferentiated P19 cells (Fig. 6), and with results from MEL18 mutant mouse embryos that exhibit ectopic *Hoxd4* gene expression (39). This suggests a possible mechanism by which YY1 binding recruits a MEL18-containing complex that maintains silencing at the *Hoxd4* locus. This is supported by the location of the YY1 binding site in an internucleosomal region, allowing YY1 to bind the repressed *Hoxd4* gene and then recruit MEL18 so as to maintain silenced chromatin at P1. Our attempts to verify whether MEL18 recruitment to P1 is decreased following YY1 knockdown using ChIP did not reveal significant results (data not shown), possibly due to the incomplete knockdown of YY1 in our experiments. Alternatively, MEL18 binding may be independent of YY1, or become so once recruited.

YY1 remains bound to P1 following *Hoxd4* activation in neurally differentiating P19 cells, and its knockdown results in further increases in *Hoxd4* transcription. However, MEL18 is no longer present at the active P1 promoter, suggesting that the release of MEL18, but not YY1, is required for gene induction. Our results further demonstrate a role for YY1 in dampening activated transcription at *Hoxd4*. Why would repressive YY1 function be required at transcriptionally active loci? Repressive HDAC and chromatin remodeling functions are required for limiting cyclical rounds of transcriptional initiation at the estrogen-receptor target gene pS2 (55). Similarly, YY1 may recruit such activities to curtail RA-induced *Hoxd4* expression. Regardless of the inhibitory mechanism, YY1 binding to P1 attenuates but does not abolish transcription and therefore could fine-tune the amount of *Hoxd4* transcript and protein levels available under specific conditions. A recent study in *Drosophila* suggests that the repressive effect of the HOX protein Ultrabithorax (UBX) on Distal-less (Dll) transcription in the limb is highly concentration dependent (56). Therefore, YY1 might be required to fine-tune the appropriate amount of HOXD4 required to repress or activate downstream target genes. Alternatively, the relief of repression seen in RA-treated cells following YY1 knockdown may be an indirect result of altered regulation at other loci.

The *Hoxd4* ARE was first characterized in non-differentiating P19 cells and in the absence of the neural enhancer (10). In this study, we investigated the role of the ARE in regulating P1 in concert with the enhancer during neural differentiation of P19 cells. We found that the ARE plays a crucial role in initiating transcription from P1 only after RA treatment, indicating that ARE activity was also dependent on the *Hoxd4* 3’ neural enhancer. Moreover, we showed binding of HOXD4 to the ARE *in vivo* by ChIP, confirming previous results obtained in EMSA (10). Interestingly, we also detected significant PBX1 recruitment to the *Hoxd4* ARE in RA-treated P19 cells, although the ARE in question does not clearly harbor a HOX-PBX binding site.
(TGATTNAT). Previous work in Drosophila embryos suggest that although DFD can interact with simple DNA-binding sites in the absence of EXD, protein-protein tethering of EXD is required to release the intrinsic DFD transactivation function which is otherwise masked by the homeodomain (34). HOXD4 might similarly tether PBX given the high degree of functional conservation among the Dfd and Hoxd4 AREs, and among HOX proteins and their cofactors (57).

Finally, we also mapped a positive regulatory element located 3’ to the transcriptional start site (Fig. 2B). This element is spanned by nucleosome N3 (Fig. 1C) and is therefore more accessible to transcription factor binding after RA treatment. We tried to identify the transcription factor binding to the GC-rich motif at the core promoter downstream to the transcriptional start sites. Although Sp1 has been shown to regulate the expression of several Hox genes (52,58,59), we conclude that it does not act through the GC-rich motif at P1 (data not shown). Other proteins of interest include AP-2 that has been shown to regulate Hoxa2 (12), and USF-1 (52,60) which binds Hoxb4 and Hoxb7. On the other hand, this GC-rich motif may be functionally similar to other previously described core promoter elements, such as the downstream promoter element (DPE), which have been shown to participate in initiating transcription from numerous proximal promoters (61). Further studies are needed to confirm these suggestions.

In conclusion, we have shown that multiple levels of control at P1 influence the regulation of Hoxd4 gene expression. An integration of these results with those of our earlier study (27) suggests a model for the concerted action of factor recruitment and chromatin modification or remodeling at the Hoxd4 enhancer and P1 promoter (Fig. 8). Further study will explore the mechanistic links between the enhancer and promoter that jointly specify the fine temporal and spatial patterns of Hoxd4 expression in the developing embryo.

FOOTNOTES

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FIGURE LEGENDS

Figure 1. Nucleosome positioning and remodeling at Hoxd4 P1. A) Results from MNase digestion experiments coupled to LM-PCR using radiolabeled primers O-115 and O-69 are shown schematically. The positions of digestion products are shown by the horizontal lines along with the size in bp. The resulting coordinates of N1, N2 and N3 are relative to the P1 transcriptional start site (+1). B) Undifferentiated P19 cells (-) or cells treated with RA (+) were digested with either 1u (lanes 1 and 2) or 5u (lanes 3 and 4) of MNase for 5 min, and naked DNA controls (N) were digested with 0.25 u of MNase for 5 min. Arrows denote major protections obtained by LM-PCR using primer O-115 (left panel) and O-69 (right panel), followed by gel electrophoresis. Sizes were determined by running sequencing reactions from a known source on the same gel. C) Loading controls for samples used in sections A and B. Real-time PCR was performed using gapdh-specific oligos, and PCR was terminated during log phase. Quantitative representation of the total amount of DNA is shown (left) as well as the ethidium bromide-stained gel of the PCR products. D) Upper panel: Schematic showing the positions of nucleosomes N, N2, and N3, restriction sites of Kpn I and Pvu II, and the positions of primers O-115 and O-195. Lower panels: Chromatin extracts were partially digested in vivo with different concentrations and durations of either Kpn I (left) or Pvu II (right). Following DNA purification, samples were fully digested with Eco RI for normalization. The gels show the cleavage products of Kpn I and Pvu II digests of chromatin samples extracted from untreated (-) or RA-treated (+) P19 cells. LM-PCR was performed following restriction enzyme digests using radiolabeled O-115 (for Kpn I) or O-195 (for Pvu II) followed by gel electrophoresis and autoradiography.

Figure 2. P1 deletion constructs and luciferase reporter assays. A) Schematic drawing of pXP2CL-Hoxd4P1 firefly luciferase reporter bearing the 540 bp Hoxd4 neural enhancer and Hoxd4 P1 sequences between positions -800 and +140 relative to the transcriptional start site (+1). Equals construct 3 below. B) Luciferase assay results for different promoter deletion constructs reported as relative luciferase activity over β-gal units. Untreated (-RA, black bars) or RA-treated (+RA, gray bars) P19 cells were co-transfected with deletion constructs together with RSV-βgal vector as an internal control. All experiments were repeated three times and error bars represent standard deviation of three independent experiments. NE = Hoxd4 neural enhancer.
Figure 3. Hoxd4 ARE is crucial for P1 activity and recruits HOXD4 and PBX1 in RA-treated P19 cells. A) Conservation between mouse and zebrafish of DNA sequences spanning the Hoxd4 ARE including two TAAT/ATTA HOXD4 binding sites. B) Site-directed mutation of both TAAT/ATTA motifs in a pXP2CL-Hoxd4P1 gives rise to plasmid mARE. The mutated underlined nucleotides are mutated into the sequence above shown in gray. Graph shows results of luciferase reporter assays of RA-treated P19 cells transfected either with pXP2CL-Hoxd4P1 (wtARE) or with mARE. The experiments were repeated at least twice and error bars represent SEMs. C) ChIP experiments were performed with either polyclonal anti-HOXD4 antibodies or no antibody (No Ab) as a negative control using chromatin extracts of untreated (black bars) or RA-treated. Results are presented as % input. Real-time PCR was performed using oligonucleotides specific for the Hoxd4 ARE or gapdh as a control (see Table 1) and shows HOXD4 recruitment to the ARE following RA treatment. Experiments were performed at least twice and error bars represent SEMs. D) ChIP experiments using PBX-1 polyclonal antibodies show recruitment of PBX1 to the HOXD4 ARE in RA-treated cells. E) Results from ChIP experiments using MEIS-1 polyclonal antibodies, showing that MEIS-1 is not recruited to the Hoxd4 ARE in RA-treated and untreated P19 cells.

Figure 4. Site-specific YY1 binding to Hoxd4 P1. A) Sequence comparison between Hoxd4 P1 repressory sequences and the YY1 binding site consensus sequence. The location of the YY1 binding site is shown relative to nucleosomes N1 and N2. B) EMSA using the Hoxd4 YY1 binding site as the labeled probe and nuclear extracts (NE) from untreated (-) or RA-treated (+) P19 cells. Competition with either 10x or 100x concentrations of cold probe or cold YY1 consensus oligonucleotides (cons.) inhibit complex formation, while the same concentrations of a non-specific oligonucleotides (nonsp.) does not. C) Anti-YY1 antibodies but not other unrelated antibodies (anti-RARγ, anti-PBX1 and anti-TFIID) inhibit complex formation between nuclear extracts and Hoxd4 P1 YY1 binding site.

Figure 5. YY1 represses reporter construct activity and binds to Hoxd4 P1 in vivo. A) Schematic showing mutation of Hoxd4 P1 YY1 binding site (mYY1) in construct 4 background (Fig. 2). B) Luciferase reporter assays showing increased activation of the reporter construct
containing the mutated Hoxd4 YY1 binding site as compared to the wild-type construct. Values are the averages of two independent experiments with error bars representing SEMs. C) ChIP experiments with anti-YY1 antibodies using chromatin extracts from untreated (black bars) or RA-treated (gray bars) P19 cells. Mock ChIP experiments using no antibodies (No Ab) were performed as controls. Real-time PCR was performed using primers specific for the region of the P1 YY1 binding site or gapdh as a control, and values are presented as percent of input material.

Figure 6. Knockdown of yy1 increases Hoxd4 transcription in P19 cells. A) Upper panel: Knockdown of yy1 in undifferentiated P19 cells. Western blots (WB) using anti-YY1 antibodies representing two independent yy1 knockdown experiments (sets 1 and 2) using whole cell extracts of untransfected (-) or transfected P19 cells with either pSUPER-YY1 (YY1 siRNA) or pSUPER-CTRL (ctrl siRNA) vectors. Membranes were reprobed with anti-actin antibodies as a loading control. Lower panel: Reverse transcription (RT) was performed on total RNA extracted from P19 cells used in the upper panel. PCR was performed using primers specific for Hoxd4 homeobox region (Hoxd4) before (-) and after RT, or gapdh coding region (gapdh) as a control. B) YY1 knockdown in RA-treated P19 cells. Upper and lower panels as for Fig. 6 A. Results show specific increases in Hoxd4 transcripts following YY1 knockdown. C) ChIP with MEL18 antibodies or no antibody (No Ab) as a negative control using chromatin extracts from untreated (black bars) or RA-treated (gray bars) P19 cells. Real-time PCR was performed using primers spanning the Hoxd4 YY1 binding site (Hoxd4 P1) or corresponding to a gapdh control. D) Sequential ChIP performed first with anti-YY1 antibodies followed by a second ChIP with anti-MEL18 antibodies. This was followed by real-time PCR using either Hoxd4 P1 or gapdh specific oligonucleotides. E) Western blot analysis showing the expression of MEL18 in nuclear (N) and cytoplasmic (C) fractions of P19 cells either treated (+) or untreated (-) with RA. TFIID expression was analyzed in both fractions to ensure efficient extraction of nuclear versus cytoplasmic fractions. HOXD4 protein expression was performed to confirm transcriptional activation following RA treatment.

Figure 7. A positive promoter element maps downstream of the Hoxd4 transcriptional start site. A) Luciferase reporter constructs and their activities in transfected RA-treated P19 cells. Construct 7 is the same as that presented in Fig. 2B. Results are the averages of two
independent experiments and error bars represent SEMs. B) EMSAs using either labeled probe A (containing P1 sequences +1 to +33), B (+34 to +66), or C (+1 to +66) (Table1). Nuclear extracts (NE) from untreated (-) or RA-treated (+) P19 cells were used. The arrow points to a specific DNA-protein complex. C) Competition experiments using probe B (+34 to +66).

Figure 8. A model of events at the Hoxd4 neural enhancer and P1 promoter leading to transcriptional activation. The diagram depicts the Hoxd4 P1 promoter with the three positioned nucleosomes mapped in this study, an internucleosomal YY1•MEL18 complex, the upstream ARE, the transcriptional start site (subtended arrow) and the 3’ Hoxd4 neural enhancer plus bound RXR•RAR heterodimer. Nucleosomes are present at the Hoxd4 enhancer but have not been mapped (dotted circles) and the relative position of the RXR•RAR heterodimer is arbitrary. Results from the current study and from Rastegar et al. (27) suggest the following order of events: (1) Retinoic acid is bound by retinoid receptors present at the previously characterized RARE in the Hoxd4 neural enhancer (19,24), leading to (2) recruitment of coactivators such as CBP and (3) methylation and acetylation of histone H3 on lysine 4 and lysine 9, respectively, and N-terminal acetylation of histone H4. These modifications are hallmarks of transcriptionally active chromatin and correlate with an “open” or accessible state. Recruitment of additional transcription factors and coactivators to the enhancer, perhaps in conjunction with a 5’ spreading of histone modifications (27), leads to (4) release of MEL18 from a complex with YY1 at the P1 promoter. HOXD4 subsequently binds the upstream ARE and tethers PBX to this region via protein-protein interactions, while a positively acting GC-rich element recruits an unknown factor (GC) just downstream of the transcriptional start site (5). Recruitment of coactivators such as CBP (6) leads to covalent modification of histones H3 and H4 (7) and relaxation of DNA without a change in nucleosome position. RNA polymerase II (pol II) arrives at the enhancer (8) before the promoter (27), placing Hoxd4 among a growing list of genes in which the enhancer may deliver (9) polymerase to the promoter (62). RNA polymerase II, in cooperation with the basal transcriptional machinery, now engages the sterically accessible promoter region and transcription is initiated. The continued presence of YY1 may be required to limit successive rounds of reinitiation. Note that while this chronology is experimentally validated at several points, the sequence is partly speculative.
## Table 1. Oligonucleotides used in this study.

|                      | 5’-3’                      |
|----------------------|----------------------------|
| **LM-PCR**           |                            |
| Linker oligo LM-PCR 1| GCGGTGACCCTCGGAGATCTGAC    |
| Linker oligo LM-PCR 2| GAATTCAGATC                |
| O-115                | CTTAGTCTGCTGGTAGGACCAGG    |
| O-69                 | AGACACGTTCACCTCGGGGCTCC    |
| O-195                | CCGAGCCTACCTGACCAGCTCTGA   |
| **ChIP**             | 5’-3’                      |
| Hoxd4 ARE forward    | TACTCTTCTGTGCTGCTGTC       |
| Hoxd4 ARE reverse    | TGCTTCTGCTGCTGATATG        |
| Hoxd4 P1 YY1 binding site forward | GAACTCATGCTGAGACAGG |
| Hoxd4 P1 YY1 binding site reverse  | CAGAGCAGATCCAGGC |
| gapdh NAD-binding domain forward | AACGCACCTCTCAGAC |
| gapdh NAD-binding domain reverse  | TCCAGCACATCTCAGGC |
| **EMSA**             | 5’-3’                      |
| YY1 consensus sense  | GGGGATCAGGGTCTCCTATTTGGA   |
| YY1 consensus anti-sense | GCGGAGATCTCCC |
| Hoxd4 P1 YY1 binding site sense | CTTACCTGACCAGTCCTGAAAAGCC |
| Hoxd4 P1 YY1 binding site anti-sense  | CCTGCGTTTCAAGATCGTGGAGG |
| Oligo A (1-33)       | ATGGTCTGATGCAAAAACATCTATAT |
| Oligo B (34-66)      | ATGGCCAGAGACTGAGGGCAGAGAG |
| Oligo C (1-66)       | ATGGTCTGATGCAAAAACATCTATAT |
| **RT-PCR**           | 5’-3’                      |
| Hoxd4 homeobox forward | CTACACCAGAGCAAGCTCC |
| Hoxd4 homeobox reverse | CTATAAGGTCAGGGGCTCC |
| **Site-directed mutagenesis** | 5’-3’ |
| mYY1 forward         | ACAACGAGACTCGAGCCAGCTTG   |
| mYY1 reverse         | GCCGGCTTCTACTCTGAGTCAGG   |
| **siRNA**            |                            |
| YY1 siRNA sense      | AAGAUGAGGCGUCAGAAAGdTdT    |
| YY1 siRNA antisense  | GCCUCUGGACAUCAUCUdTdT      |
| Ctrl siRNA sense     | CCUCUCUGUGAGAAATCCAGAdTdT  |
| Ctrl siRNA antisense | UGGAGGUUCACGAGGdTdT        |
Figure 4

A

Hoxd4 P1
YY1 consensus

-190 TACCTGCACCATCTCTGAAAG -170
CGCCATNTNCN

B

|        | cold probe 10x | cold probe 100x | cons. 10x | cons. 100x | nonsp. 10x | nonsp. 100x |
|--------|---------------|-----------------|-----------|------------|------------|-------------|
| RA     | -             | -               | +         | -          | -          | +           |
| NE     | -             | +               | +         | +          | +          | +           |

C

|        | Anti-YY1 | Anti-RARα | Anti-PBX1 | Anti-TFIIID |
|--------|----------|-----------|-----------|-------------|
| RA     | -        | -         | +         | -           |
| NE     | -        | +         | +         | +           |
Figure 5

(A) Construct 4

(B) Bar graph showing relative Luc/β-Gal levels

(C) Relative % input for YY1 and No Ab treatments.

IP: α-YY1
PCR: Hoxd4 P1

IP: α-YY1
PCR: gapdh
Figure 6

A

WB

\[
\begin{align*}
Y|Y|RNA & \quad - \quad \text{set1} \\
\text{ctrl} & \quad \text{ctrlRNA} & \quad \text{set2} \\
\hline
\alpha-YY1 & \quad \text{set1} \\
\alpha-\text{actin} & \quad \text{set2} \\
\end{align*}
\]

B

WB

\[
\begin{align*}
Y|Y|RNA & \quad - \quad \text{set1} \\
\text{ctrl} & \quad \text{ctrlRNA} & \quad \text{set2} \\
\hline
\alpha-YY1 & \quad \text{set1} \\
\alpha-\text{actin} & \quad \text{set2} \\
\end{align*}
\]

RT-PCR

\[
\begin{align*}
\text{RT} & \quad \text{Y|Y|RNA} \\
\text{ctrl} & \quad \text{ctrlRNA} & \quad \text{set1} \\
\hline
\text{Hoxd4} & \quad \text{set1} \\
gapdh & \quad \text{set2} \\
\end{align*}
\]

C

\[
\begin{align*}
\% \text{ input} & \quad \text{MEL18} \\
\text{P19 monolayers} & \quad \text{No Ab} \\
\text{P19 + RA} & \\
\end{align*}
\]

D

\[
\begin{align*}
\% \text{ input} & \quad \text{YY1-MEL18} \\
\text{IP:} \alpha-\text{YY1} / \alpha-\text{MEL18} & \quad \text{PCR:} \text{Hoxd4 P1} \\
\text{P19 monolayers} & \quad \text{No Ab} \\
\text{P19 + RA} & \\
\end{align*}
\]

E

\[
\begin{align*}
\text{RA} & \quad - \quad + \\
\alpha-\text{MEL18} & \quad \text{set1} \\
\alpha-\text{TFIIH} & \quad \text{set2} \\
\alpha-\text{HOXD4} & \quad \text{set1} \\
\text{N} & \quad \text{C} \\
\end{align*}
\]
Figure 7
Figure 8

promoter       neural enhancer/RARE

1. Retinoic acid
2. CBP recruited
3. H3-Ac/Me, H4-Ac

4. MEL18 released
5. HOXD4, PBX & GC recruited
6. CBP recruited
7. H3-Me, Ac/H4-Ac
10. Pol II recruitment
Interplay between chromatin and trans-acting factors regulating the Hoxd4 promoter
during neural differentiation
Laila Kobrossy, Mojgan Rastegar and Mark Featherstone

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