Hoxd13 and Hoxa13 Directly Control the Expression of the EphA7 Ephrin Tyrosine Kinase Receptor in Developing Limbs

Valentina Salsi* and Vincenzo Zappavigna†

From the †Department of Animal Biology, University of Modena and Reggio Emilia, Via G. Campi 213/d, Modena 41100, Italy and the ‡Department of Cell and Developmental Biology, Cornell University Weill Medical School, New York, New York 10021

Hoxa and Hoxd genes, related to the Drosophila Abd-B gene, display regionally restricted expression patterns and are necessary for the formation of the limb skeletal elements. Hox genes encode transcription factors, which are supposed to control the expression of a series of downstream target genes, whose nature has remained largely elusive. Several genes were identified that are differentially expressed in relation to Hox gene activity; few studies, however, explored their direct regulation by Hox proteins. Ephrin tyrosine kinase receptors and ephrins have been proposed as Hox targets, and recently, evidence was gained for their role in limb development. The expression of the EphA7 gene in developing limbs was shown to correlate with the expression of Hoxa13 and Hoxd13; however, its direct regulation by these genes has never been assessed. We have characterized the EphA7 promoter region and show that it contains multiple binding sites for paralog group 13 Hox proteins. We found that one of these sites is bound in vivo by HOXA13 and HOXD13 and by endogenous Hoxd13 in developing mouse limbs. Moreover, we show that HOXD13 and HOXA13 activate transcription from the EphA7 promoter and that a mutation of the HOXA13/HOXD13 binding site was sufficient to abolish activation. Conversely, the HOXD13(I47L) mutation, identified in patients displaying a novel brachydactyly-polydactyly syndrome, does not bind to in vivo, and fails to transactivate the EphA7 promoter. These results establish that EphA7 is a direct downstream target of Hoxd13 and Hoxa13 during limb development, thus providing further insight into the regulatory networks that control limb patterning.

Transcription factors belonging to the HOX family of homeodomain-containing proteins control cell fates and regional identities along the primary body and limb axes (1, 2). Hox genes that are related to the Drosophila Abd-B gene and are located at the 5′-end of the Hoxa and Hoxd clusters (paralogous groups 9–13) display regionally restricted expression patterns in the developing limbs (3–5). Targeted mutagenesis and overexpression of Abd-B-related Hoxa and Hoxd genes showed that their function in limb development is to control the size, shape, and number of specific bones by regulating processes such as mesenchymal cell aggregation, chondrification, and ossification (6–8) (reviewed in Refs. 5 and 9). The genetic pathways in which they act, however, are still poorly characterized. In particular, the identity of their downstream effector genes remains still elusive (10). Indeed, whereas HOX proteins have been shown to bind specific DNA sequences and are supposed to regulate overlapping sets of target genes, only a few of them have been isolated. Target genes for HOX proteins have been identified using various approaches, including microarray hybridization screenings and candidate gene analysis (11–16). Few studies, however, have shown direct regulation by HOX proteins on the promoters of their putative downstream target genes.

Ephrins and ephrin tyrosine kinase receptors (Eph)2 have been recurrently indicated as HOX downstream target genes (12, 17–19). Eph and ephrins are expressed in various regions of the vertebrate embryo in dynamic patterns, and they were found to play crucial roles in the control of cell shape, cell migration, cell sorting, wiring of neurons in the nervous system, and the formation of boundaries between structures (reviewed in Refs. 20 and 21). Recently, experimental evidence has been gained for a role of Eph-ephrin signaling in limb development. Overexpression in developing chick limbs of ephrin A2 has been shown to disrupt limb cartilage morphogenesis causing digit bifurcations and syndactyly (22). Similarly, heterozygous ephrin B1 null female mice have been reported to display preaxial polydactyly and syndactyly (23). Finally, the EphA7 gene, which is expressed at embryonic day 13.5 (E13.5) in the perichondrium of the mesenchymal condensations of the phalanges, was shown to be significantly down-regulated in the forelimbs of Hoxa13-/− mice. Its expression, however, was not completely absent, suggesting that the transcription of this ephrin receptor gene might be under the control of more than one paralogous group 13 Hox protein (19). Indeed, we could show that the misexpression of Hoxd13 by retroviral infection of developing chick limbs leads to a marked increase of EphA7 expression in the phalangeal mesenchymal condensations, indicating that Hoxd13 might regulate EphA7 transcription as well. Interestingly, in the same set of experiments, we found that the misexpression of a mutant HOXD13 protein, HOXD13(I47L), did not result in an increase of EphA7 expression (24). The HOXD13(I47L) mutation, identified in patients showing a novel brachydactyly-polydactyly syndrome, represents a single amino acid substitution, involving residue 47 of the homeodomain that is located within the recognition helix. This substitution was found to alter rather than abolish DNA binding, since it selectively impaired the ability of HOXD13 to recognize one of its two different classes of consensus binding sequences (24). This finding prompted us to speculate that the I47L mutation, via a reduction of the repertoire of potential sites recognized by the HOXD13 protein, would cause a partial or complete failure to regulate a subset of the genes normally controlled by HOXD13. Since EphA7 turned out to be one of these genes, we decided to further investigate its regulation by HOXD13. In particular, we wanted to ascertain whether EphA7 is indeed a direct target of HOXD13 and/or HOXA13 and whether the
nature of the regulatory sequences mediating HOXD13 regulation would explain the lack of control by the mutated HOXD13(I47L) protein.

In this report, we show that the EphA7 promoter region contains multiple potential binding sites for HOX paralog group 13 proteins. We found, however, that only one of these sites is bound in vivo by the HOXA13 and HOXD13 proteins. A mutation of this evolutionarily conserved site was sufficient to abolish the transcriptional activation of the EphA7 promoter by HOXA13 and HOXD13. We moreover found that this EphA7 Hox group 13 site is not bound by the HOXD13(I47L) mutated protein both in vivo and in vitro, thus providing a molecular basis for the lack of up-regulation of EphA7 expression by HOXD13(I47L) in developing limbs. Our results thus establish that EphA7 is a direct downstream target of the Hoxd13 and Hoxa13 proteins during limb development, providing additional evidence that Hox gene products directly control the aggregation properties of limb mesenchymal cells by regulating the ephrin receptor-ephrin signaling system.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The EphA7 (−497 and −2000 bp promoter sequences were obtained by PCR amplification from genomic NIH-3T3 DNA using the following primers: EphA7(−497)for, 5′-GGTTATCCGACCTGTCGAGGCTCTAAC-3′; EphA7(−2000)for, 5′-GGTGGACAACTAGTGACCTTGCAGT-3′. Amplified fragments were verified by sequencing, digested with XhoI and HindIII, and cloned into the XhoI and HindIII sites of the pXP-luciferase vector. A 552-bp fragment, carrying a mutation in HOXD13 binding site 3, was obtained by reverse mutated primer, EphA7revm (5′-CGACTGCAG-3′), generated by cloning a Klenow-filled HindIII cDNA fragment into the EcoRI and HindIII sites of the pXP-luciferase vector. A 552-bp fragment is a direct downstream target of the EphA7 promoter region. The EphA7−497, EphA7−2000, EphA7revm, EphA7S1rev, EphA7S2rev, EphA7S3for, EphA7S4/5rev, EphA7rev, EphA7S2for, EphA7S3for, and EphA7S4/5for were verified by sequencing.

Electrophoretic Mobility Shift Assay—The GST-HOXD13HD and GST-HOXD13(I47L)HD fusion proteins were produced in Escherichia coli, purified according to established methods, and analyzed by SDS-PAGE and Coomassie staining. The purified proteins were diluted in 13 μl of 50 mM HEPES, 20 mM KCl, 2 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol and preincubated with 100 ng poly(dI-dC) in a total volume of 20 μl of 1X binding buffer (0.1 M KCl, 2 mM MgCl₂, 4 mM spermidine, 0.1 mg/ml bovine serum albumin) for 15 min on ice. The labeled fragments containing a consensus binding site for HOXD13 or the EphA7 HOXD13 binding site 3 were obtained by annealing of the following oligonucleotides: HOXBSA/B (24); EphA7BSA, 5′-TCGACCTAATTGATATTTGGAGGTGGAGCAC-3′; EphA7BSB, 5′-TCGCGTGCACCTAATTGATATTTGGAGGTGGAGCAC-3′. 30,000 cpm of the labeled probes were added to the samples and incubated for 30 min on ice. Reactions were separated on a 6% polyacrylamide gel in 0.5% TBE, dried, and exposed to Eastman Kodak Co. X-OmatR film at ~80 °C.

Cell Culture, Transfection, and Transduction—NIH3T3 mouse fibroblasts and C3H10T1/2 mouse embryonic fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Cellbio) supplemented with 10% fetal calf serum (Cellbio), 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfections in NIH3T3 cells were carried out by CaPO₄ precipitation (25). In a typical experiment, 12 μg of reporter plasmid, 2.5–5.0 μg of expression construct, and 0.1 μg of CMV-β-galactosidase (Clontech) as internal control were used per 6-cm dish. Forty-eight hours after transfection, cells were washed, lysed, and assayed for luciferase and β-galactosidase expression (26). Each transfection was done in duplicate in the same experiment, and the plotted luciferase activities represent the average of 3–6 different experiments. To transiently express HA-HOXD13, HA-HOXA13, or HA-HOXD13(I47L) in NIH3T3 cells, we transfected 5 μg of the corresponding expression vector per 10-cm dish. To transduce C3H10T1/2 cells, viral stocks of the LXIΔN and LHOXD13ΔN retroviral expression constructs were produced by transient transfection of Eco-Phoenix cells as described previously (27). The viral supernatant was added to a subconfluent culture of C3H10 T1/2 cells in the presence of 0.8 μg/ml Polybrene; infection was performed twice, initially for 6 h and then overnight. Cells were washed, and medium was replaced. HOXD13 transient expression in NIH3T3 and stable expression in C3H10T1/2 were detected by Western blot analysis using anti-HA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies. An antibody against the NFy-B nuclear transcription factor (kind gift of C. Imbriano) was used as a loading control. Immunostained bands were detected with a chemiluminescence system (Amersham Biosciences).

Chromatin Immunoprecipitations—Formaldehyde cross-linking and chromatin immunoprecipitations were performed as described in Ref. 28, with the following modifications: NIH3T3 and C3H10T1/2 cells were fixed for 15 min with 1% formaldehyde at room temperature, and the reaction was quenched with 0.125 mM glycine in 1X PBS for 5 min. The cross-linked material was sonicated 15 × 25 s to obtain 500–1000-bp fragments, and the immunoprecipitations were performed with 10 μl of protein G-agarose (KPL), blocked twice with 1 μg/ml salmon sperm DNA (Sigma), and 1 μg/ml bovine serum albumin, first for 2 h and then overnight. The chromatin was precleared by adding 20 μl of protein G-agarose for 2 h and incubated with 5 μg of anti-HA polyclonal antibody (sc-7392; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or 5 μg of a purified rabbit polyclonal anti-HOXD13 antibody or with 5 μg of anti-FLAG (F3165; Sigma) or anti-GAL4 antibody (sc-577; Santa Cruz Biotechnology) as a control. The incubation was performed overnight at 4 °C. Chromatin immunoprecipitation on E13.5 mouse limbs and brain was performed according to the protocol of the P. Farnham laboratory (genomcenter.ucdavis.edu/farnham/farnham), with the following modifications. To disaggregate tissues, the samples were homogenized 20 times with a Dounce B homogenizer and then frozen-thawed 20 times before sonicating 60 × 30 s; the immunoprecipitations were carried out as described above. PCR amplifications were performed using the following primers: EphA7S1for, 5′-CTTTGGTGAATCTCCGAGCACT-3′; EphA7S2rev, 5′-TGCACTTCTTACGACACGGTA-3′; EphA7S2for, 5′-GTCCAGGATTTGGAACCTTTTGTGCAA-3′; EphA7S2rev, 5′-CCATTTGAAATTTCCACTTACAGC-3′; EphA7S3for, 5′-GGACCGCCTACCCCGCTGCCG-3′; EphA7rev, 5′-GCTGCTTCGAAGTCTTCCGACTGC-3′; EphA7S4/5for, 5′-TCGGAGACTTTGCAAGGCAAAC-3′; EphA7S4/5rev, 5′-CAACATCTGGCAGATGAGGGT-3′; EphA7Cfor, 5′-GGAATCATCATGCTTCGAGGCGTTGAA-3′; EphA7Crev, 5′-TCTGGATCCTTCTCCTATGCTTCG-3′.

RT-PCR Analysis—RNA from NIH3T3 and C3H10T1/2 cells was extracted using the RNaseasy kit (Qiagen) according to the manufacturer’s protocol. Synthesis of cDNA was done starting from 3 μg of RNA using the SSII reverse transcriptase kit (Invitrogen). Semiquantitative
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PCR was performed with the following oligonucleotides: EphA7RTfor, 5′-TCTACACACGACTGGTGAAAATAC-3′; EphA7RTrev, 5′-CGCTGAGCTTGGTCTAATTTTGCTT-3′. Glyceraldehyde-3-phosphate dehydrogenase control RT-PCR was done using standard oligonucleotides.

RESULTS

The 5′ Region of the Mouse EphA7 Gene Contains Several Potential Binding Sites for the HOXD13 Protein—To identify possible HOXD13-dependent regulatory elements within the transcriptional control regions of the EphA7 gene, we analyzed genomic sequences upstream to the putative transcription start site of the EphA7 gene. An interspecies comparison using the UCSC Genome Browser (29) was made, with the idea that sequence conservation might highlight relevant regulatory regions. We found that at distances greater than 5 kb 5′ to the putative transcription start site of EphA7, the degree of sequence conservation drops significantly, whereas the highest degree of interspecies similarity is found within a region of ~2 kb upstream of the start site (Fig. 1A), suggesting that regulatory elements crucial for EphA7 expression might be confined to this region. The MatInspector software (30, 31) was subsequently employed to scan the 5 kb upstream region of EphA7 for possible HOXD13 binding sites. We previously showed, through optimal DNA binding site selection experiments, that the HOXD13 protein has an equal preference for two types of sites, one having TTAT and the other having TTAC as core consensus sequence (24). Based on our previous results, a binding site matrix was generated, using the MatInspector program (30). We identified five putative HOXD13 binding sites within the 5′ region of the EphA7 gene having a matrix similarity of 1.0 (Fig. 1A). Four of them have TTAT as a core consensus sequence, and one has TTAC. Moreover, four of the identified sites are located within the proximal region of the promoter (sites 2–5; Fig. 1A), and three of these (sites 3–5) are located downstream from the putative transcription start site (Fig. 1, A and B). A sequence alignment of these putative HOXD13 DNA binding sites with the corresponding available genomic sequences from different species revealed that of the five sites identified within the mouse genome, only site 3 displays a high degree of conservation (Fig. 1A, bottom part). These results indicated that the sequences upstream to and in the vicinity of the presumed transcription start site of EphA7 contain several putative HOXD13 binding sites that match the optimal consensus binding sequence determined in vitro, suggesting that Hoxd13 and possibly also Hoxa13 may directly associate with these sites to regulate EphA7 expression.

The EphA7 Promoter Is Bound in Vivo by HOXD13—We then set out to verify which of the putative sites was actually bound in vivo by HOXD13. For this purpose, we exogenously expressed a HA-tagged HOXD13 protein both stably and transiently in two different mouse cell lines of mesenchymal derivation, C3H10T1/2 and NIH3T3 fibroblasts. In C3H10T1/2 cells, stable expression was achieved by retrovirus-mediated gene transfer (Fig. 2A, left), whereas transient expression in NIH3T3 cells was obtained using a plasmid expression vector (Fig. 2A, right). We found that the exogenous expression of HOXD13 could efficiently activate transcription of the endogenous EphA7 gene both in C3H10T1/2 and in NIH3T3 cells (Fig. 2B), indicating that the expressed HOXD13 protein is functional and that the chosen cell backgrounds are permissive for activation of EphA7 transcription by HOXD13 and therefore represent suitable models for studying the interactions of HOXD13 with the EphA7 promoter in vivo.

To verify the binding in vivo of HOXD13 to the candidate binding sites within the EphA7 promoter, we used the chromatin formaldehyde cross-linking and immunoprecipitation (ChIP) technique (32). Cross-linked and sonicated chromatin from HA-HOXD13-expressing C3H10T1/2 cells was immunoprecipitated using an anti-HA antibody (αHA) and was analyzed by PCR for the presence of the five putative HOXD13 binding sites. In addition, as a control, we tested for the presence of a region of 263 bp located at ~6000 bp (control site; Fig. 3A). As shown in Fig. 3B, the immunoprecipitated chromatin showed a substantial enrichment only of the sequence including site 3, indicating that only site 3 was efficiently bound in vivo by HOXD13. No enrichment was detected for the remaining sites, as well as for the control site (Fig. 3B). Thus, of the five sites matching the optimal DNA-binding consensus sequence of HOXD13, which could hence all be potentially bound by HOXD13, only site 3 turned out to be actually contacted in vivo by HOXD13.

We then wanted to test whether also endogenous Hoxd13 is bound in vivo, in the developing limb, to site 3 of the EphA7 promoter. To this end, chromatin was prepared from E13.5 mouse fore and hind limbs and immunoprecipitated using a αHoxd13 antibody. As a control, immunoprecipitation experiments were performed using chromatin extracted from E13.5 mouse brain, a tissue that does not express Hoxd13 (33). The immunoprecipitated limb chromatin showed a significant enrichment of the fragment containing site 3 (Fig. 3C). No enrichment of the site 3-containing sequence was observed in the control brain chromatin (Fig. 3C), as well as no enrichment of the control sequence (C site; Fig. 3C) was detected. Thus, these data confirm that site 3 is bound in vivo during mouse limb development at a stage when both Hoxd13 and EphA7 are coexpressed in the perichondrium of the digit condensations.

The HOXD13(I47L)-mutated Protein Does Not Bind to the EphA7 Promoter—We previously reported that the HOXD13(I47L) mutation, identified in patients showing a novel brachydactyly-polydactyly syndrome, caused a selective impairment of the DNA binding potential of HOXD13 (24). Indeed, the HOXD13(I47L) mutant protein, unlike its wild type counterpart, proved to be unable to up-regulate the expression of the endogenous EphA7 gene, if overexpressed in developing chick limbs, suggesting that it is unable to bind to the EphA7 promoter. To test this assumption, we verified by chromatin immunoprecipitation whether HOXD13(I47L) was able to bind site 3 of the EphA7 promoter in vivo. HA-HOXD13, HA-HOXA13, or HA-HOXD13(I47L) was transiently expressed in NIH3T3 cells, and their chromatin was tested by immunoprecipitation using an αHA antibody. As in the case of HA-HOXD13-expressing C3H10T1/2 cells, the immunoprecipitated chromatin from NIH3T3 cells expressing HA-HOXD13, showed a significant enrichment only of the sequence containing site 3 (Fig. 4A; data not shown), indicating that also in this cell background only site 3 of the EphA7 promoter was occupied in vivo by HOXD13. Similarly, Hoxa13, which was previously reported to be a candidate regulator of EphA7 in limb development (19), was found to be bound in vivo to EphA7 site 3 (Fig. 4A). In contrast, chromatin immunoprecipitated from NIH3T3 cells expressing HA-HOXD13(I47L) showed no enrichment for the sequence containing site 3 (Fig. 4A), indicating that HOXD13(I47L) does not bind to this site in vivo.

We then verified the binding of HOXD13 and HOXD13(I47L) to EphA7 site 3 in vitro in electrophoretic mobility shift assays. As shown in Fig. 4B, HOXD13 bound efficiently the site 3 sequence, whereas HOXD13(I47L) bound site 3 weakly, only at higher concentrations. A control site, having a TTAC core sequence, was bound comparably well by HOXD13 and HOXD13(I47L) (Fig. 4B). Taken together, these results show that HOXD13(I47L), unlike its wild type counterpart, does not bind to EphA7 site 3 both in vivo and in vitro, providing a molecular basis for the lack of regulation of EphA7 expression by HOXD13(I47L).
HOXD13 and HOXA13 Activate Transcription from the EphA7 Promoter, whereas HOXD13(I47L) Does Not—We next wanted to assess whether the EphA7 promoter region could mediate transcriptional activation by HOXD13 or HOXA13. For this purpose, we generated a luciferase reporter construct containing a fragment ranging from −2000 to +55 of the EphA7 genomic sequence (pXP-EphA7−2000; Fig. 5A), which comprises the promoter region with the highest degree of evolutionary conservation and includes site 3 (Fig. 1A). NIH3T3 cells were
transiently co-transfected with pXP-EphA7(−2000) and increasing amounts of SV40-driven constructs expressing HA-HOXD13, HA-HOXA13, or HA-HOXD13/I47L. As shown in Fig. 4B, both HOXD13 and HOXA13 significantly increased the basal reporter activity, whereas the expression of HOXD13/I47L led only to a modest activation of the reporter. We then generated a deletion construct (pXP-EphA7(−497); Fig. 5A) containing a fragment from −497 to +55 of the EphA7 promoter, comprising site 3. As shown in Fig. 4C, both HOXD13 and HOXA13 could efficiently activate the pXP-EphA7(−497) reporter basal activity to levels comparable with those obtained with the pXP-EphA7(−2000) reporter. Conversely, the co-expression of HOXD13/I47L induced only a minor activation of the pXP-EphA7(−497) reporter (Fig. 5B). Finally, we mutated the sequence of site 3 within the context of the pXP-EphA7(−497) reporter (pXP-EphA7(−497m); Fig. 5A), changing it from ATATTATTGG into ATAGGCGTGG, to alter the core consensus sequence for HOXD13 binding. As shown in Fig. 5C, the expression of HOXD13, HOXA13, or HOXD13/I47L had virtually no effect on the (pXP-EphA7(−497m)) reporter basal activity, indicating that a mutation of site 3 was sufficient to abolish transactivation by HOXD13 and HOXA13 on the EphA7 promoter.

These results confirm the importance of site 3 in mediating transcriptional activation of the EphA7 promoter by paralog group 13 Hox proteins. These data moreover show that, unlike HOXD13 and HOXA13, HOXD13/I47L is unable to bind to EphA7 site 3 and thus to regulate the EphA7 promoter. This result is in accordance with the inability of HOXD13/I47L to up-regulate endogenous EphA7 in developing limbs (24).

**DISCUSSION**

The nature of the downstream target genes for Hox proteins has remained largely elusive. Whereas various approaches have led to the identification of a number of genes that are differentially expressed in relation to Hox gene activity, few studies have addressed the direct regulation of target gene promoters by Hox transcription factors. In this work, we have analyzed the promoter of the mouse EphA7 gene for direct regulation by the HOXD13 and HOXA13 proteins. Recent reports have pointed to the EphA7 ephrin tyrosine kinase receptor gene as a possible downstream target of Hoxa13 and Hoxd13 protein function. Indeed, EphA7 is co-expressed in developing limbs with Hoxd13 and Hoxa13 in the perichondrium of the mesenchymal condensations of the phalanges, and the expression of EphA7 was found to be significantly reduced in Hoxa13+/− and Hoxd13−/− mutant mice (19). Additionally, the over-expression of Hoxd13 by retroviral infection of developing chick limbs was shown to induce a marked increase of EphA7 expression in the phalangeal mesenchymal condensations, indicating that EphA7 transcription might be regulated by Hoxd13 as well (24).

**The genomic region upstream to EphA7 contains multiple binding sites for group 13 Hox proteins**—Since the EphA7 promoter had never been previously characterized, we analyzed the genomic sequence upstream to the transcription start site of EphA7 for the presence of putative Hoxd13 binding sites using an *in silico* approach. Binding site selection studies have shown that the *Drosophila* AbdB protein has a preference for sites with a TTAT core sequence and that Abd-B-related vertebrate Hox proteins preferentially bind to sites with a TTAT or TTAC core sequence (34, 35). In accordance, we previously reported that Hoxd13 binds equally well to two distinct sites, one TTATTTGG with TTAT as core binding sequence and the other TTTCAGGAG with TTAC as core element (24). Similarly, Shen et al. (35) reported a Hoxd13 consensus binding site with a TTAC core sequence. Based on these data, we generated a position weight matrix (36) describing the Hoxd13 binding site and exploited it to identify Hoxd13 binding sites within the EphA7 promoter region. Five different putative Hoxd13 binding sites were thus identified, matching with the reported Hoxd13 consensus binding sequences. Four of the putative Hoxd13 binding sites (sites 2–5) were found to match with the TTAT-based consensus site, whereas only one (site 1) matched with the TTAC-based consensus. Interestingly, four of the identified sites are localized to the most conserved part of the EphA7 promoter region (−2000 bp to +500 from the transcription start site), and three of them (sites 3–5) map 3′ and in close proximity to the transcription start site.

**Hoxd13 and Hoxa13 bind in vivo to a single evolutionarily conserved site within the EphA7 promoter**—The presence of multiple sites matching the optimal DNA binding sequence of Hoxd13 would in theory imply that Hoxd13 and possibly Hoxa13 occupy concomitantly all or most of these sites to regulate EphA7 expression. We found, however, by ChIP that of the five putative Hoxd13 binding sites within the EphA7 promoter, only site 3 (TTATTTGG) is actually bound in vivo by Hoxd13 as well as by Hoxa13, indicating that site 3 is the only functionally relevant paralog group 13 Hox binding site within the EphA7 promoter. In accordance with this result, a mutation of site 3 in the context of the EphA7 promoter was sufficient to abolish the transcriptional activation of EphA7 by Hoxa13 and Hoxd13.

Significantly, site 3 is the only Hox group 13 binding sequence within the EphA7 regulatory region that shows a substantial degree of evolutionary conservation, being identical in six of seven species compared. The remaining sites, conversely, display a considerably lower degree of conservation, with site 1 being present only in the mouse genome. Thus, evolutionary conservation within the EphA7 promoter, of the site 3 paralog group 13 regulatory element is consistent with its functional relevance in the regulation of EphA7 expression.

The sequence of EphA7 site 3 (TTATTTGG) matches with the optimal DNA binding sequence determined in site selection experiments (TTATTTGG), indicating that the binding specificity of HOXD13 *in vitro* corresponds to that observed *in vivo*. It can be therefore concluded that paralog group 13 Hox proteins apparently do not require co-factors,
The HOXD13(I47L) Mutated Protein Displays a Selective Impairment of Its DNA Binding Ability in Vivo—We previously reported that a missense mutation that substitutes leucine for isoleucine at position 47 which alter and/or increase their intrinsic DNA binding specificity, to bind in vivo to their regulatory element within the EphA7 promoter. Similar results were reported for the Bmp2 and Bmp7 regulatory elements, which were shown to be bound by Hoxa13 in vivo at discrete sequences that are closely related to the optimal DNA-binding consensus sequence for paralog group 13 Hox proteins (14).

Our finding that of the multiple potential binding sites for group 13 Hox proteins within the mouse EphA7 promoter only a single site is actually bound in vivo is in contrast with a previously proposed model for Q50 homeodomain protein binding in vivo (37). This model was suggested on the basis of the in vivo binding behavior of the Drosophila Eve and Ftz proteins. According to it, Q50 homeodomain proteins, unlike the majority of transcription factors, bind, in the absence of cofactors, at uniform levels to many sites throughout the length of their target genes (38, 39). Our results and the results reported by Knosp et al. (14) for the Hoxa13 protein conversely indicate that vertebrate group 13 Hox proteins bind to discrete sites within specific regulatory regions of their direct target genes, behaving in this respect as “conventional” transcription factors. The discrepancy between the binding behavior of Eve/Ftz and group 13 Hox proteins may derive from the possible differences in the intrinsic properties of these proteins or, more likely, from the different approaches used to detect in vivo binding. Indeed, whereas the in vivo binding by Eve and Ftz was examined by UV photocross-linking, a technique that allows efficient mapping of binding in vivo only to relatively large DNA fragments, possibly including many different binding sites, in this and in the study by Knosp et al. (14), formaldehyde cross-linking ChIP was used, which allows the detection of binding at a higher resolution (40). In fact, we were able to clearly discriminate between bound (site 3) and unbound (sites 4/5) sites located only 165 bp apart.

Several mechanisms could be envisaged to explain the lack of binding in vivo by Hoxd13 and Hoxa13 to sites matching the optimal DNA-binding consensus sequence for paralog group 13 Hox proteins. However, the chromatin configuration or the binding of other transcription factors at or in close proximity to these binding sites are the most likely causes for their lack of accessibility and thus of their functional ineffectiveness. Indeed, the DNA context was shown to play an important role in differentiating functional binding sites from mere physical binding sites (41, 42).
(I47L) of the HOXD13 homeodomain, identified in patients showing a novel brachydactyly-polydactyly syndrome, causes a selective impairment of the ability of HOXD13 to bind DNA. HOXD13(I47L) was found to be unable to recognize binding sites having TTAT or TAAT as the core sequence, whereas sites with a TTAC or TAAC core were bound with the same efficiency as wild type HOXD13 (24). The EphA7 promoter contains one potential Hoxd13 binding site with a TTAC core (site 1, TTTACG) and could therefore in theory be regulated by HOXD13(I47L) as well. We previously observed, however, that misexpression of the HOXD13(I47L) mutant protein, as opposed to wild type
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Hoxd13, did not result in the up-regulation of endogenous EphA7 in developing chick limbs (24), suggesting that Hoxd13(147L) is unable to bind the EphA7 promoter. Indeed, our results indicate that the only functionally relevant HOX group 13 binding site within the EphA7 promoter is site 3 (TTATGT), with a TTAT core sequence, which in principle should not be recognized by HOXD13(147L). Using ChIP analysis, we could confirm that EphA7 site 3 is not bound in vivo by HOXD13(147L). HOXD13(147L) proved furthermore to be unable to activate transcription from the EphA7 promoter. Finally, the only potential binding site with a TTAC core, site 1, turned out to be the least evolutionarily conserved group 13 Hox binding site within the potential binding site with a TTAC core, site 1, turned out to be the least functionally relevant HOX group 13 binding site within the EphA7 promoter, thus further supporting the assumption that it is functionally irrelevant. In conclusion, our results showing that EphA7 is a direct target of Hoxd13 allow us to confirm that the selective loss of its DNA binding ability leads to a failure of HOXD13(147L) to regulate some of the direct target genes that are normally controlled by HOXD13, providing a molecular basis for the pathogenesis of the novel brachydactyly-syndactyly syndrome produced by this mutation.

EphA7 Is a Direct Downstream Target of Hoxd13 and Hoxa13 in Limb Development—Signaling between Eph and their ephrin ligands plays a fundamental role in controlling several developmental processes and has been proposed to be a downstream effector of a number of Hox genes in some of these (reviewed in Ref. 43). Evidence for a role of Eph-ephrin signaling also in limb patterning has been recently accumulating (19, 22, 23). During limb development, segmentation and bifurcation processes shape blocks of condensed mesenchyme to generate the prepattern of the limb skeletal elements. These processes are particularly important in the autopod, which is composed by numerous skeletal elements such as the phalanges of the digits and the carpal/tarsal bones. Eph-ephrin signaling in the limbs was proposed to be involved in the regulation of intercellular affinity, which is a prerequisite for the correct condensation of limb mesenchyme (22, 23). Thus, positional identity of the mesenchymal cells, as provided by the expression of Hox genes, may be implemented by differential variations in cellular affinities, which affect the pattern of limb mesenchymal condensations. Hoxa13 and Hoxd13 probably play crucial and partially overlapping roles in this process. Indeed, in Hoxa13<sup>−/−</sup> and Hoxd13<sup>−/−</sup> mutant mice, the fore and hind limb most anterior digits are absent and the prechondrogenic condensations of the autopod are not formed at all (45). Fromental-Ramain, C., Warot, X., Messadecq, N., LeMeur, M., Dolle, P., and Champigny, P. (1996) Development 122, 1449–1466.

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