THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 4, pp. 1992–1999, January 27, 2006

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

Received for publication, October 5, 2005, and in revised form, November 10, 2005 Published, JBC Papers in Press, November 28, 2005, DOI 10.1074/jbc.M510900200

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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(147L) 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

* This work was supported by grants from the Italian Association for Cancer Research, and the Italian Association for the Study of Malformations (to V. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: Eph, ephrin tyrosine kinase receptor(s); E13.5, embryonic day 13.5; HA, hemagglutinin; αHA, anti-HA; ChIP, chromatin formaldehyde cross-linking and immunoprecipitation; RT, reverse transcription.

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′-GTTATCCGACCTGCTGAGGCTGCTAAC-3′; EphA7(−2000)for, 5′-GTGGCAATCTAGGATGTTGAGACCTCA-3′; EphA7rev, 5′-GCTGCCTGCAAGTCTCCGACTGC-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 was PCR-amplified from the cloned template to generate a reporter construct (EphA7−497m), carrying a mutation in HOXD13 binding site 3, by using a reverse mutated primer, EphA7revm (5′-CGACTGCAGACCGGCCGCTTGCTCCACACACTCCACGCCTATCAATTAG-3′). The retroviral construct expressing HOXD13 was generated cloning a HOXD13 Klenow-filled XhoI-HindIII cDNA fragment in frame with the HA tag into the Klenow-filled EcoRI site of the LXIΔN retroviral vector. The pSGHA-HOXA13 expression construct was generated by cloning a Klenow-filled HindIII cDNA fragment into the Klenow-filled EcoRI site of the pSG5 expression vector (Stratagene). The expression vectors for HA-HOXD13, HA-HOXD13(I47L), and the GST-homeodomain fusion proteins (HOXD13HD and HOXD13-(I47L)HD) were generated as described previously (24).

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 δ-buffer (20% glycerol, 20 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol) and preincubated with 100 ng poly(dI-dC) in a total volume of 20 μl of 1× binding buffer (0.1 M KCl, 2 mM $MgCl_2$, 4 mM spermidine, 0.1 mg/ml bovine serum albumin) for 15 min on ice. ^{32}P-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′-TCGACCCTAATTGATATTATTGGAGTGTGGAGCAC-3′; EphA7BSB, 5′-TCGCGTGCTCCACACTCCAATATCAATTAGGG-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 (Celbio) supplemented with 10% fetal calf serum (Celbio), 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfections in NIH3T3 cells were carried out by $CaPO_4$ 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 LHOXD13IΔN 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; incubation 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 M glycine in 1× 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 (genomecenter.ucdavis.edu/farnham/farnham), with the following modifications. To disaggregate tissues, the samples were homogenized 20 times with a Dounce B homogenizer and then freezed-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′-CTTTGTGTAATCCGAGCACTAC-3′; EphA7S1rev, 5′-TGCATCTTTACGACACGGTA-3′; EphA7S2for, 5′-GTCCGAGGTTGAACTTTTTGTCCA-3′; EphA7S2rev, 5′-CCACTTGAATTCACCCAATCCTAGC-3′; EphA7S3for, 5′-GCAACCGACTCCGCTCGGC-3′; EphA7rev, 5′-GCTGCCTGCAAGTCTCCGACTGC-3′; EphA7S4/5for, 5′-TCGGAGACTTGCAGGCAGCAA-3′; EphA7S4/5rev, 5′-CAACCATGGTGCATGAGCAGGT-3′; EphA7Cfor, 5′-GGAAGTACCATGCTTGAAAGTGA-3′; EphA7Crev, 5′-TCTGGATCCTTCTCATTCTCTGGA-3′.

RT-PCR Analysis—RNA from NIH3T3 and C3H10T1/2 cells was extracted using the RNeasy 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

PCR was performed with the following oligonucleotides: EphA7RTfor, 5′-TCTACACCACGACTGGTGGAAAAA-3′; EphA7RTrev, 5′-CCGCTCGAGCTTGGGTTTCGAATCATTTTGTCT-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. 1*A*), 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 MatInd 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. 1*A*). 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. 1*A*), 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. 1*A*, *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. 2*A*, *left*), whereas transient expression in NIH3T3 cells was obtained using a plasmid expression vector (Fig. 2*A*, *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. 2*B*), 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. 3*A*). As shown in Fig. 3*B*, 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. 3*B*). 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. 3*C*). No enrichment of the site 3-containing sequence was observed in the control brain chromatin (Fig. 3*C*), as well as no enrichment of the control sequence (C site; Fig. 3*C*) 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. 4*A*; 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. 4*A*). In contrast, chromatin immunoprecipitated from NIH3T3 cells expressing HA-HOXD13(I47L) showed no enrichment for the sequence containing site 3 (Fig. 4*A*), 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. 4*B*, 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. 4*B*). 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).

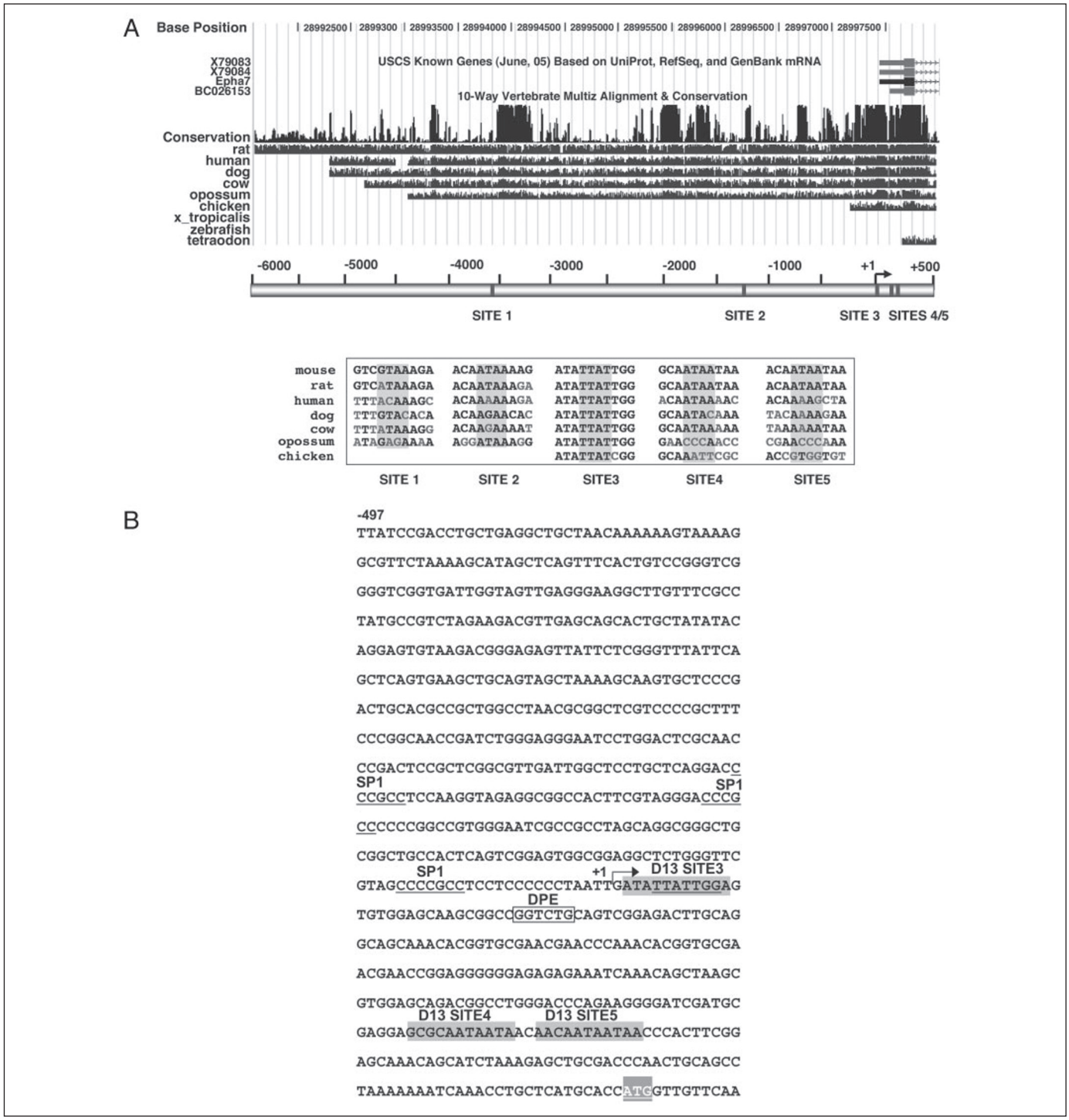

FIGURE 1. **The 5′ region of the mouse *EphA7* gene contains potential HOXD13 binding sites.** *A*, interspecies comparison of the *EphA7* genomic sequences spanning from −6000 bp upstream to +500 bp downstream to the putative transcription start site. Alignments to the Chr. 4:28992000–28998500 mouse genomic sequence were performed using the UCSC Genome Browser (29). The highest density of interspecies conservation is found within a region of 2 kb upstream to the transcription start site (indicated by an *arrow* in the diagram *below*). The degree of sequence conservation drops significantly for distances greater than 5 kb upstream to the start site of transcription. A schematic representation of the *EphA7* genomic region is shown *below*. An *arrow* indicates the transcription start site. Five different putative HOX binding sites (sites 1–5, indicated by *black boxes*) were found within the analyzed genomic region using the MatInspector software and a HOXD13 binding site matrix generated by the MatInd program (30). The interspecies conservation of the five sites is shown *below* the *schematic diagram*. *Gray boxes* highlight the consensus core binding sequences. *B*, nucleotide sequence of the *EphA7* proximal promoter region (−497 to +286). The putative transcription start site (+1) is indicated by an *arrow*. The *gray boxes* highlight HOXD13 binding sites. The conserved HOXD13 binding site 3 is *underlined*. *DPE*, downstream promoter element. *SP1*, binding sites for the Sp1 transcription factor. The EphA7 protein translation start site (*ATG*) is indicated.

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. 5*A*), which comprises the promoter region with the highest degree of evolutionary conservation and includes site 3 (Fig. 1*A*). NIH3T3 cells were

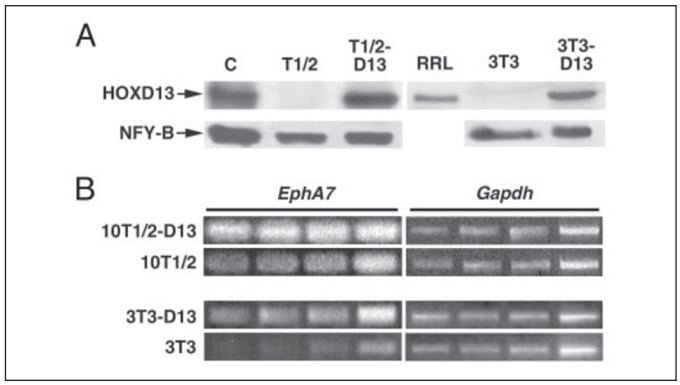

FIGURE 2. **The exogenous expression of HOXD13 up-regulates *EphA7* transcription in NIH3T3 and C3H10T1/2 cells.** The HA-tagged HOXD13 protein was exogenously expressed both stably and transiently, in C3H10T1/2 and NIH3T3 fibroblasts, respectively. In C3H10T1/2 cells, stable expression was achieved by retrovirus-mediated gene transfer, and transient expression in NIH3T3 cells was obtained using a plasmid expression vector. *A*, Western blots showing the expression of HOXD13 in nuclear extracts from NIH3T3 cells or C3H10T1/2 cells, transfected with HOXD13 expression vectors (3T3-D13; T1/2-D13), or from control cell lines (3T3; T1/2). *C*, control Phoenix packaging cell line expressing HOXD13; *RRL*, *in vitro* transcribed/translated HOXD13 using rabbit reticulocite lysates; *NFY-B*, an antibody against the NFY-B nuclear transcription factor was used as a loading control. *B*, semiquantitative RT-PCR analysis of *EphA7* and control *Gapdh* mRNA expression in cells expressing HOXD13 (3T3-D13; T1/2-D13) or in control cells (3T3; T1/2). Aliquots of the PCRs at increasing cycles were loaded to show a linear detection range.

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. 4*B*, 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. 5*A*) containing a fragment from −497 to +55 of the *EphA7* promoter, comprising site 3. As shown in Fig. 4*C*, 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. 5*B*). Finally, we mutated the sequence of site 3 within the context of the pXP-*EphA7*(−497) reporter (pXP-*EphA7*(−497m); Fig. 5*A*), changing it from ATATTATTGG into ATAGGCGTGG, to alter the core consensus sequence for HOXD13 binding. As shown in Fig. 5*C*, 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*$^{-/-}$ mutant mice (19). Additionally, the overexpression 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 TTTTAT-TGG with TTAT as core binding sequence and the other TTTACGAG 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 (TTATTG) 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 (TTATTGG) matches with the optimal DNA binding sequence determined in site selection experiments (TTTATTGG), 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,

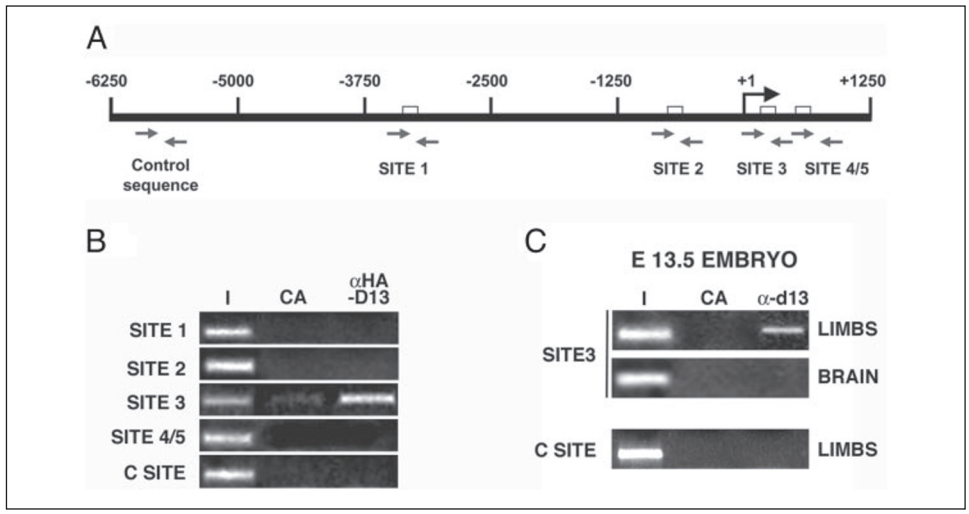

FIGURE 3. **HOXD13 binds *in vivo* to the *EphA7* promoter.** *A*, schematic representation of the *EphA7* promoter showing the position of the identified putative HOXD13 binding sites. The *arrows* indicate the primers used in ChIP assays to PCR-amplify genomic regions containing the HOXD13 binding sites or a control region (C site). *B*, ChIP on C3H10T1/2 cells expressing HOXD13 using αHA antibodies. The enrichment was analyzed by PCR using oligonucleotides that amplify genomic regions of 174 bp (site 1), 297 bp (site 2), 245 bp (site 3), or 221 bp (site 4/5) within the mouse *EphA7* promoter. No enrichment of the genomic regions containing sites 1, 2, and 4/5 or of the control site was detected even after 41 cycles of amplification. PCRs were performed in triplicate; a representative set is shown. *C*, ChIP on pooled fore and hind limbs or on brain tissue, dissected from E13.5 mouse embryos, using an anti-Hoxd13 (αHoxd13) antiserum. PCRs were performed in triplicate; representative experiments are shown. No enrichment was observed with immunoprecipitated brain chromatin even after 42 cycles of amplification, as well as no enrichment was detected for the control site (C site). *I*, input chromatin; *CA*, non-specific control antibody.

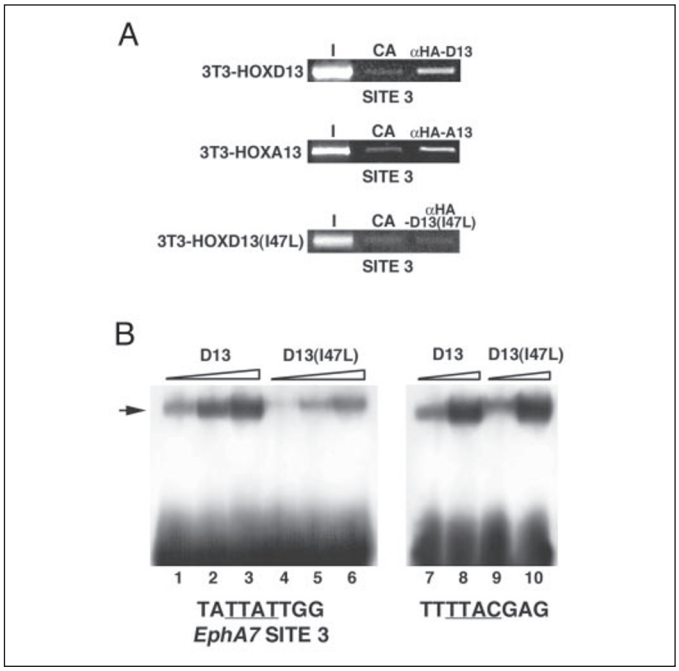

FIGURE 4. **HOXD13(I47L) fails to bind EphA7 site 3 both *in vivo* and *in vitro*.** *A*, ChIP analysis of HOXD13, HOXA13, or HOXD13(I47L) binding to *EphA7* site 3. Shown are chromatin immunoprecipitations on NIH3T3 cells transiently expressing HA-HOXA13, HA-HOXD13, or HA-HOXD13(I47L) using αHA. The enrichment was analyzed by PCR using oligonucleotides that amplify a genomic region spanning site 3 (Fig. 3*A*). PCRs were performed in triplicate; representative experiments are shown. No enrichment was detected with the chromatin from cells expressing HA-HOXD13(I47L) after 38 cycles of amplification. *CA*, nonspecific control antibody. *I*, input chromatin. *B*, electrophoretic mobility shift assay (*EMSA*) using oligonucleotide probes containing *EphA7* site 3 (*left panel*, *lanes 1–6*) or the TTTTACGAG HOXD13 DNA-binding consensus sequence (*right panel*, *lanes 7–10*). Increasing amounts of bacterially expressed, purified GST-HOXD13HD (*lanes 1–3*, *7*, and *8*), or GST-HOXD13(I47L) (*lanes 4–6*, *9*, and *10*) proteins were used. Retarded complexes are indicated by an *arrow*.

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 sequence for paralog group 13 Hox proteins. However, 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).

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

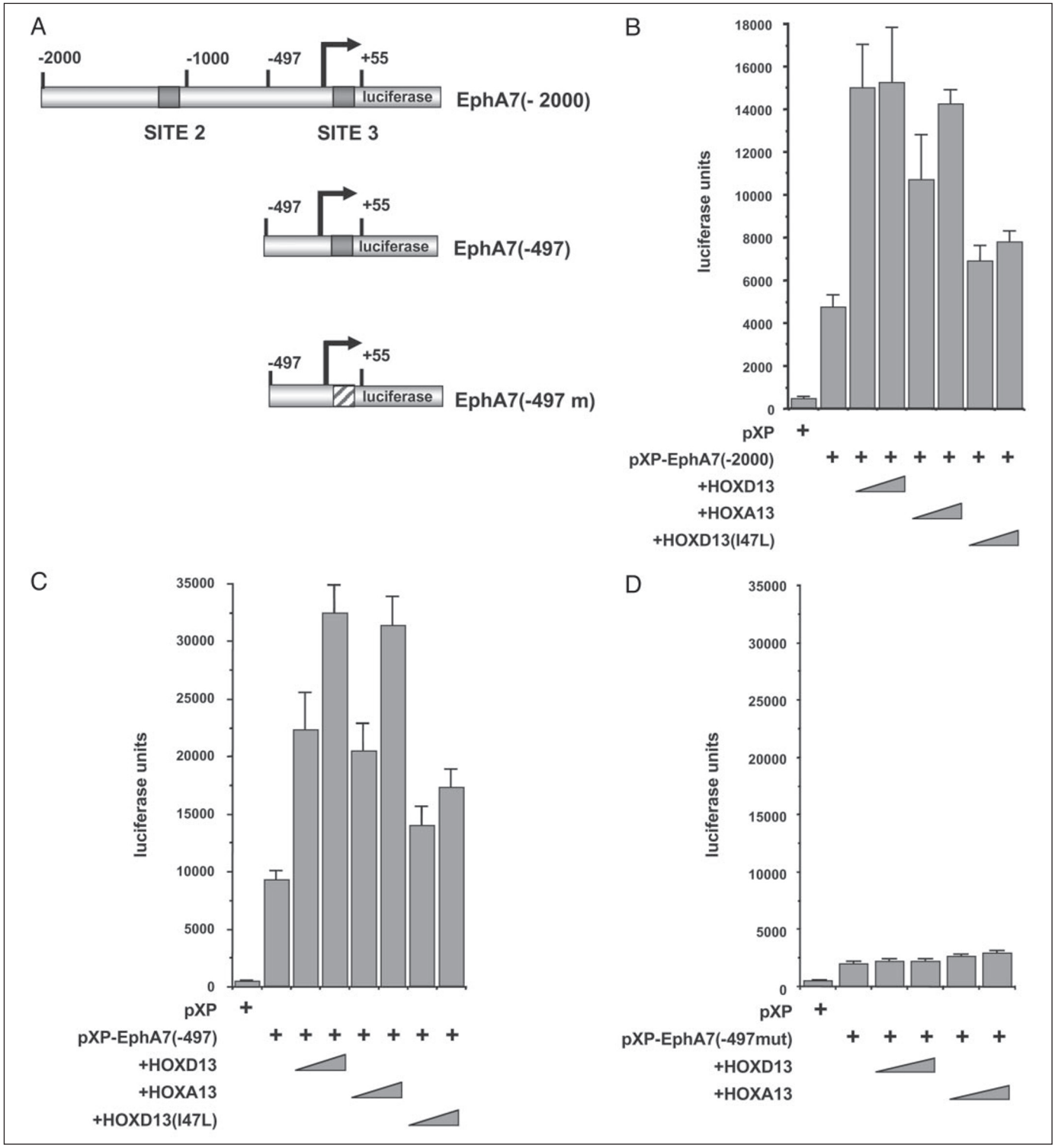

FIGURE 5. **HO_XD13 and HOXA13 activate transcription from the murine *EphA7* promoter.** NIH3T3 cells were transiently transfected with 2.5 or 5 μg of expressing constructs for HOXD13, HOXA13, or HOXD13(I47L) and 12 μg of the indicated reporter constructs. *A*, schematic representation of the reporter constructs used in transfection assays. The positions of sites 2 and 3 are indicated. A *striped box* in the *EphA7*(−497m) constructs indicates the mutation of site 3. *B*, luciferase activity, in arbitrary units, assayed from cells transfected with the *EphA7*(−2000) reporter and expression constructs for the indicated proteins. *C*, luciferase activity assayed from cells transfected with the *EphA7*(−497) reporter and expression constructs for the indicated proteins. *D*, luciferase activity assayed from cells transfected with the *EphA7*(−497m) reporter and expression constructs for the indicated proteins. *pXP*, promoterless luciferase reporter vector. *Bars* represent the mean luciferase activity ± S.E. of at least four independent experiments.

(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

HOXD13, did not result in the up-regulation of endogenous *EphA7* in developing chick limbs (24), suggesting that HOXD13(I47L) 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 (TTATTG), with a TTAT core sequence, which in principle should not be recognized by HOXD13(I47L). Using ChIP analysis, we could confirm that *EphA7* site 3 is not bound *in vivo* by HOXD13(I47L). HOXD13(I47L) 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 *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(I47L) 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 $Hoxa13^{-/-}$ mutant mice, the fore and hind limb most anterior digits are absent and the prechondrogenic condensations that give rise to the carpal, metacarpal, tarsal, and metatarsal bones are not properly formed. Analogously, $Hoxd13^{-/-}$ mutant mice display a significant growth retardation of several autopodal cartilage elements and a selective lack of bony elements that develop late in wild type mice (44). In *Hoxa13* and *Hoxd13* double homozygous mutant mice, the mesenchymal condensations of the autopod are not formed at all (45). Moreover, the misexpression of *Hoxa13* in developing chick limbs was shown to alter mesenchymal cell-cell interactions, further underscoring the role of group 13 *Hox* genes in the regulation of genetic pathways controlling aggregation and sorting properties of the distal autopodal mesenchyme (6). Our data show that both Hoxd13 and Hoxa13 bind to the *EphA7* promoter *in vivo* and activate transcription from it. They moreover show that endogenous Hoxd13 is bound to the *EphA7* promoter in developing mouse limbs at E13.5, a stage when both *Hoxd13* and *EphA7* are co-expressed in the perichondrium of the digit condensations. Thus, taken together, our results establish that *Hoxd13* and *Hoxa13* directly control the expression of *EphA7* in the developing autopod, providing further evidence that *Hox* gene products directly control the aggregation adhesion properties of the mesenchymal cells within the limbs by regulating the ephrin/ephrin receptor signaling system.

Acknowledgments—We thank Dr. A. Kuroiwa for the kind gift of anti-Hoxd13 antibodies, Prof. Alexis Grande for providing the LXIΔN retroviral vector, and Dr. Silvia Ferrari for help in the pXP-EphA7(−2000) construction.

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