Alx4 Binding to LEF-1 Regulates N-CAM Promoter Activity*

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During murine embryogenesis, expression of the paired-like homeodomain protein Alx4 is restricted to tissues whose development depends on the expression of lymphoid enhancer factor-1 (LEF-1). Given the defects seen in hair follicle development in both LEF-1 and Alx4 knockout and mutant animals and the overlapping expression patterns, we predicted that LEF-1 and Alx4 might form physical complexes. We demonstrate here the interaction between LEF-1 and Alx4. This interaction is mediated through a specific proline-rich domain in the N-terminal region of Alx4 and requires the DNA-binding domain (HMG-box) of LEF-1. We also demonstrate that LEF-1 and Alx4 can bind simultaneously to adjacent sites on the neural cell adhesion molecule (N-CAM) promoter and that this binding alters N-CAM promoter activity. Furthermore, when expressed in primary mammary stromal cells, Alx4 decreases the expression of endogenous N-CAM protein. These results reveal a potential mechanism that gives rise to mesenchymal-specific activities of LEF-1.

The patterning of many developing tissues and organs during embryogenesis is mediated by inductive processes between opposing epithelial and mesenchymal cell layers (1). Signaling between these layers governs organogenesis from initiation of tissue development to terminal differentiation of organ-specific cell types. The early stages of organogenesis are marked by the appearance of local epithelial thickenings followed by condensations of adjacent mesenchymal cells. This process is driven by a series of inductive reciprocal signals traveling between epithelial and mesenchymal cell compartments and resulting in the stepwise determination of both tissue components and, ultimately, formation of the adult organ.

Development of teeth, whiskers, hair follicles, and mammary glands represent well studied examples of tissues that arise from these reciprocal signaling processes (2–4). Despite specialized structures and functions, common signaling cascades are required for the generation of these distinct tissues. The cascades are initiated and/or mediated by soluble factors such as the Wnt (5, 6), hedgehog (Hh) (7–9), fibroblast growth factor (Fgf) (10), and bone morphogenic protein (BMP) families (11, 12) of morphogens. In addition, development of the tissues critically depends on the expression and activity of a number of transcription factors associated with these signal transduction pathways.

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One critical factor, lymphoid enhancer-binding factor (LEF-1)† (13) is a member of the high mobility group (HMG)-box family of proteins, which includes the closely related factors Tcf-1, -2, -3, and -4. Although the expression and activity of members of this family overlap, LEF-1-null mice die shortly after birth, exhibiting (among other defects) a conspicuous absence of hair, teeth, whiskers, and mammary glands (14, 15).

Forced expression of LEF-1 in mice under the control of the keratin-14 (K14) promoter causes abnormalities in the positioning and orientation of hair follicles as well as ectopic development of teeth (16). A requirement for the control of activity for other aspects of the pathways signaling through LEF-1 was demonstrated when K14-dependent expression of an activated version of the LEF-1-binding protein (β-catenin) resulted in de novo hair follicle development and, ultimately, malignant transformation of epithelial cells in the hair follicle (17).

LEF-1 is a sequence-specific (5′-CCTTG(A/T)(A/T)-3′) DNA-binding protein whose activity is modulated via cooperative interactions with non-DNA-binding cofactors such as β-catenin (18–22), AML, ALY (14, 23–26), and CBP/p300 (29). The N terminus of LEF-1 mediates direct binding to β-catenin, thereby stimulating transcription in response to Wnt signaling (18, 20, 21, 30, 31). In the absence of Wnt signaling, CBP binds LEF-1, lowering its affinity for β-catenin by acetylating a conserved lysine in the β-catenin-binding domain (29). The protein ALY interacts with the proline-rich domain of LEF-1 and with AML-1 (another TCR-binding protein) to stimulate the function of the T-cell receptor enhancer (23, 24, 32). Factors binding to LEF-1 (such as the co-repressor, Groucho) result in the formation of transcriptional repressor complexes (26–28). Thus, apart from its ability to alter the conformation of DNA itself (33), LEF-1 plays an architectural role in the assembly of multiprotein complexes (30, 34), thereby facilitating the integration of multiple signal transduction pathways in the control of transcription.

Proline-rich regions mediate protein-protein interactions between several classes of factors. We and others recently isolated a paired-like homeodomain protein, Alx4 (35, 36), that harbors an N-terminal proline-rich motif predicted to mediate complex formation with other proteins. During murine embryogenesis, Alx4 expression is restricted to mesenchymal cells in developing bones, limbs, hair, whiskers, teeth, and mammary tissues (35, 37). The Alx4-mutant mouse strain, Strong’s luxoid (Intb), exhibits limb defects, cranio-facial abnormalities, temporary dorsal alopecia, and polydactyly (38, 39). Likewise, targeted deletion of Alx4 on a C57Bl/6 background resulted in mice with multiple abnormalities including polydactyly, dorsal alopecia, cranio-facial defects, and defects in body wall closure (36).

† The abbreviations used are: LEF-1, lymphoid enhancer factor-1; CAT, chloramphenicol acetyltransferase; HMG, high mobility group; N-CAM, neural cell adhesion molecule; GST, glutathione S-transferase; HA, hemagglutinin; EMSA, electrophoretic mobility shift assay.
We demonstrated previously that Alx4 and LEF-1 expression overlap spatially and temporally in many of these same tissues during mouse embryogenesis (37). Because LEF-1 activity is required for the formation of tissues expressing Alx4, we hypothesized that LEF-1 interacts with Alx4 to regulate transcription of target genes. We demonstrate here the interaction between Alx4 and LEF-1 and show further that these two factors bind simultaneously to adjacent sites in the promoter region of the neural cell adhesion molecule, N-CAM. In addition, LEF-1 and Alx4 alter transcription from the N-CAM promoter, and Alx4 inhibits the expression of N-CAM protein when expressed in primary mammary mesenchymal cells.

MATERIALS AND METHODS

Plasmids, Constructs, and Antibodies— Murine LEF-1-1 HA cDNA and α-LEF-1 rabbit polyclonal antisera were kindly provided by Dr. R. Grosschedl (Gene Center and Institute of Biochemistry, University of Munich, Germany). Myc-tagged LEF-1 was generated by inserting a Myc oligo (5'-CTCGAGCAGAAGATCTTCTGGAAGATCTTGAGGTACC-3') in place of the HA tag. LEF-1 mutant plasmids (ΔHMGB and Δβcat) were a kind gift from Dr. L. Attisano (Department of Medical Biophysics, University of Toronto, Canada) (40). Expression vectors for HA-tagged Alx4 were subcloned into pCMV Alx4 (37). The GST fusion protein construct of Alx4 was generated by restriction digestion of pcDNA3-HA-Alx4 (BamHI/SmaI) and subsequently cloned into BamHI/SmaI sites of the pGEX-2T vector (41). The mutant Alx4αD expression plasmid was a gift from Dr. R. Wisdom (Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, TN). The pCAGGS-α-LEF-1 was a kind gift of C. Goridis (Center d’Immunologie National de la Sante, Marseille-Luminy, France). Additional deletion constructs were made by cutting at specified restriction sites and religating the vector. Sheep α-LEF-1 antisera was obtained from Bionostics, Inc. (Toronto, Ontario). Monoclonal antibodies against Alx4 were described previously (37). Two monoclonal antibodies, the mouse antibodies, the mouse hybridoma (12C5) and the rat anti-HA (Roche Molecular Biochemicals) were used to detect the HA tag. The monoclonal antibody to N-CAM (5B8), developed by Thomas M. Jessel and Jane Dodd, was obtained from the Developmental Studies Hybridoma Bank (maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA). The hybridoma 9E10 was used to generate antisera against the Myc epitope. The Alx4 adenovirus was constructed using the bacterial recombination system described by He et al. (42). Briefly, HA-tagged Alx4 was ligated into the multiple cloning site of pCMV-AdTrack, and recombination with the pAdEasy vector was facilitated in BJ5183 cells. The recombinant adenoviral genome was then transfected into 293 cells, and it recovered viral particles used for subsequent rounds of amplification in 293 cells.

GST Binding Assay— GST fusion proteins were expressed in bacteria and purified on glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer’s instructions as we have described previously (41). An equivalent amount (1 μg) of purified GST or GST-Alx4αD fusion protein bound to glutathione beads was incubated with COS-1 nuclear lysates containing LEF-1 HA protein and was rocked for 2 h at 4 °C. The glutathione beads were washed five times in a Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 120 mM NaCl, 50 mM Tris, pH 8.0), and bound proteins were eluted by boiling in 2× SDS buffer for 5 min before loading onto 10% SDS-polyacrylamide gels.

Immunoprecipitation and Western Blot— COS-1 cells were transfected with various expression constructs using the DEAE-dextran method. Cells were lysed 48-h post-transfection, and lysates were prepared in a Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 120 mM NaCl, 50 mM Tris, pH 8.0) and immunoprecipitated with sheep anti-LEF-1 antisera. 20 μl of protein G-Sepharose beads were then added and incubated for a further 2 h. Immunocomplexes were washed five times with ice-cold Nonidet P-40 lysis buffer and separated on 8% reducing SDS-PAGE gels. Following transfer to nitrocellulose, membrane blots were blocked overnight at 4 °C in phosphate-buffered saline containing 5% skim milk powder. Blots were incubated at room temperature for 2 h in a 1:1000 dilution of primary antibody followed by a 1-h incubation with a 1:8000 dilution of the appropriate secondary horseradish peroxidase antibody. Blots were developed with the ECL fluorescent detection kit according to the manufacturer’s instructions (Amersham Biosciences).

Cell Culture, DNA Transfections, CAT, and β-galactosidase Assays— Human C33A cells were maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum (Sigma) as well as penicillin and streptomycin (Invitrogen). For CAT assays, C33A cells were transfected by the calcium phosphate method. Cells were harvested after 48 h, and CAT assays were performed as described previously (37). CAT activity was normalized relative to the internal control β-galactosidase. In all experiments, the total amount of transfected DNA was kept constant by including the appropriate empty expression vector. Data shown are from representative experiments that were done in triplicate, and the error bars indicate the standard error of the mean.

Electrophoretic Mobility Shift Assays (EMSAs)— Nuclear lysates were prepared as described previously (43). Gel shift reactions contained 15 μg Tris (pH 7.5), 75 mM NaCl, 1.5 mM EDTA, 0.3% Nonidet P-40, 0.8 μg of poly(dI-dC), 4 mM spermidine, 4 mM spermine, 1.5 mM dithiothreitol, and 7.5% glycerol. After incubation at room temperature for 5 min, 32P-labeled probe was added, the mixture was incubated at room temperature for 20 min, and then antibodies were added and incubated for an additional 10 min. Samples were separated on 4% polyacrylamide gels that contained 0.5× Tris borate/EDTA. The sequence of the gel shift probes are as follows (top strand): LEF1 site, 5′-GGTCCCAAACCTTTGAAATCGAAGAGGATCTGTAG-3′; p1 site, 5′-CTTTTCTCCCCAAATTATATTAAAAACCGTTCAA-3′; N-CAM probe: LEF-1 and p1 sites, 5′-GACCAAATTGAAAATCGAAGAGGATCTGTAGAAT-3′; TCCCCCAATTATATTAAAAACCGTTCAA-3′. Immunohistochemistry— Immunohistochemistry was performed on paraffin-embedded sections (6 μm) of parafinembeddedfixed embryos (E15.5). Rabbit antiserum against LEF-1 was used at 1:400 dilution. Supernatants of monoclonal antibodies against Alx4 and N-CAM were used undiluted. Secondary antibodies, α-rabbit-Texas Red and α-mouse-FITC (Jackson ImmunoResearch Laboratories, Inc.), were used at 1:1000 dilution. Immunofluorescence confocal microscopy was carried out as described previously (44) on a Bio-Rad MRC-600 confocal fluorescence microscope.

RESULTS

Alx4 Stably Interacts with LEF-1— We determined previously that expression of the paired-like homeodomain protein Alx4 is restricted to mesenchymal cells in a number of tissues whose development is critically dependent on the expression of the HMG-box protein, LEF-1 (37). The presence of a proline-rich SH3-protein binding motif2 in the N-terminal region of Alx4 and the ability of LEF-1 to directly bind other transcription factors on DNA further suggested that LEF-1 and Alx4 might form co-complexes. This hypothesis was initially tested in vitro using GST pull-down assays (Fig. 1). As illustrated, transiently expressed full-length HA-tagged LEF-1 in COS-1 cells binds specifically to GST-Alx4 but not to GST alone.

2 K. Boras, unpublished data.
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Binding of LEF-1 to Alx4 was further characterized in co-immunoprecipitation experiments (Fig. 2). COS-1 cells were transiently co-transfected with the expression plasmids for HA-tagged Alx4 and HA-tagged LEF-1. LEF-1 was immunoprecipitated from nuclear lysates using an α-LEF-1 polyclonal antibody, and co-immunoprecipitated HA-Alx4 was detected by Western analysis using the α-HA antibody, 12CA5 (Fig. 2, lane 2). When co-expressed, LEF-1 mediates co-immunoprecipitation of Alx4, confirming the results of the in vitro pull-down assay.

The polyproline motif in the N-terminal region of Alx4 (PQPPTQPQPPPAPP, amino acids 92–107) bears a striking similarity to motifs in factors that mediate protein-protein interactions (46). This region was examined for its possible role in Alx4 interaction with LEF-1 (Fig. 2). Constructs expressing various deletions of Alx4 (Fig. 2A) were co-transfected with the LEF-1-myc expression vector. All mutants of Alx4 are co-expressed with LEF-1 in the nucleus (Fig. 2A). LEF-1 was immunoprecipitated using sheep polyclonal antisera directed against the β-catenin binding site (Δβcat) or the DNA-binding HMG-box (ΔHMG) were co-transfected with the Alx4 expression vector. Whole cell lysates were immunoprecipitated with a sheep α-LEF-1 polyclonal antibody, and the associated Alx4 was detected using an α-Alx4 antibody (top panel). Immunoprecipitated LEF-1 was visualized by re-probing the blot with a rabbit α-HA antibody (second panel). The expression levels of Alx4 (Fig. 2B, middle panel). Thus, the SH3 protein-binding motif of Alx4 is required for its interaction with LEF-1. The C-terminal proline-rich domain (amino acids 294–327), the highly conserved paired-tail motif (amino acids 375–391) (47), as well as the sequence N-terminal to the SH3 protein-binding motif in Alx4 are not required for strong association with LEF-1.

Fig. 2C reveals further that, distinct from β-catenin, Alx4 appears to bind to the domain responsible for LEF-1 binding to DNA, the HMG-box. Specifically, wild-type LEF-1 or LEF-1 that lacks the β-catenin binding site (Δβcat) or the HMG-box (ΔHMG) were co-transfected with Alx4. Immunoprecipitation of wild-type LEF-1 or the Δβcat mutant co-immunoprecipitated Alx4. However, the ΔHMG mutant, which was expressed at levels similar to wild-type LEF-1, failed to immunoprecipitate Alx4. Alx4 and LEF-1 Regulate N-CAM Promoter Activity—Mice that are mutant for either Alx4 or LEF-1 exhibit a number of similar developmental defects, including that of hair follicles (15, 38). Furthermore, we showed previously that expression of these two factors overlaps significantly (37). N-CAM expression during hair follicle development has been shown previously to be very dynamic, being detectable in mesenchymal cells and/or epithelial cells at distinct stages of the hair follicle cycle (48, 49). As we demonstrate in Fig. 3, N-CAM is expressed in an overlapping pattern with LEF-1 in the in-
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vaginating epithelial cells and is excluded from the dermal papilla where Alx4 is expressed. Regulation of N-CAM expression by these factors is further supported by the observation that noggin-dependent induction of LEF-1 coincidentally increases N-CAM expression (50). Furthermore, our inspection of the promoter region of N-CAM revealed two putative LEF-1 consensus binding sequences (CTTTG(A/T)(A/T) (14, 33, 34); see Fig. 4A), one of which (position −589) is adjacent to an AT-rich sequence encoding the palindromic P1 paired-like homeodomain binding sequence, TAATATAA (51, 52). Alx4 monomers have been shown to bind with high affinity to a portion of this site, specifically TAATTT (53).

Thus, using a series of 5′-deletion N-CAM promoter constructs, we determined the effect of LEF-1 expression on N-CAM promoter activity (Fig. 4B). In the absence of LEF-1, N-CAM promoter activity increased when the sequence between −647 and −1003 bp (ΔNde) was deleted. When an additional 58 bp were removed (ΔHinf0) the region encoding the distal LEF-1 consensus sequence, promoter activity decreased to half of the activity seen for the ΔSpI construct. Further deletion decreased activity to levels that approach background. Co-transfection of the ΔSpI N-CAM promoter-CAT construct with 2.0 μg of the LEF-1 expression vector increased N-CAM promoter activity by 2-fold. This increase was also evident for the ΔNde mutant where both LEF-1 binding sites were intact. However, further deletion of 58 bp (ΔHinf0) to just upstream of the putative P1 binding site (with the deletion of the distal LEF-1 consensus binding site) prevented LEF-1 from activating the N-CAM promoter, indicating that the upstream sequence was the principal site for LEF-1-dependent N-CAM promoter activation. Furthermore, transfection with the LEF-1 DNA-binding mutant (ΔHMG) and a dominant-negative LEF-1 mutant unable to bind β-catenin (Δβcat) did not activate the N-CAM promoter (Fig. 4C).

We next examined the effect of N-CAM promoter activity in the presence of expressed LEF-1 and Alx4 (Fig. 4D). Cells were transiently transfected with the N-CAM ΔNde-CAT reporter construct together with increasing amounts of Alx4 or LEF-1 expression plasmids. Expression of LEF-1 strongly activated the N-CAM promoter up to 12-fold over basal activity. Alx4 weakly stimulated promoter activity in a dose-dependent manner. When the two expression plasmids were co-transfected, low levels of Alx4 reproducibly enhanced LEF-1-dependent N-CAM promoter activation. However, as higher levels of Alx4 were expressed, LEF-1-dependent activation was repressed (Fig. 4D). Transfection of the DNA-binding-deficient mutant derived from lef0 mice (Alx4Δtet) failed to alter LEF-1-dependent N-CAM activity (Fig. 4E).

Alx4 and LEF-1 Bind Simultaneously to the N-CAM Promoter—Given the proximity of the LEF-1 and Alx4 binding site consensus sequences and the ability of these proteins to interact with one another, we examined whether the two proteins bind these sites on the N-CAM promoter simultaneously (Fig. 5). EMSAs were performed using a labeled double-stranded oligonucleotide probe encoding the sequence between −595 and −535 of the N-CAM promoter (Fig. 5A). As the lower panel of Fig. 5A demonstrates, nuclear lysates containing exogenous Myc-tagged LEF-1 produced a novel complex (lane 1, C4), which was specifically supershifted with the α-Myc monoclonal antibody, 9E10 (lane 2, C2). Competition with 200-fold excess cold N-CAM oligo abrogated the LEF1-DNA complexes (lanes 3 and 4). As we and others have shown previously (36, 37), Alx4 binds only weakly to DNA encoding a paired-like homeodomain consensus sequence (lane 5). However, stabilization of Alx4 on DNA was apparent with the addition of the α-Alx4 antibody (lane 6, C3). As with LEF-1, competition with cold N-CAM oligo abrogated all Alx4-DNA complexes (lanes 7 and 8). Mutation of either the LEF-1 or P1 DNA sites also abrogated shifts seen in Fig. 5A (data not shown). These data demonstrate that an intact LEF-1 binding site is required for LEF1 binding and that an intact Alx4 binding site is required for Alx4 binding. Control lysates did not bind to the radiolabeled N-CAM oligo (lanes 9–15).

In the presence of LEF-1, enhanced binding of Alx4 to DNA was observed. Specifically, novel complexes are seen when Alx4 and LEF-1 are co-expressed (Fig. 5B, lane 1). Supershifts with α-Myc and α-Alx4 antibodies (lane 2) verified the presence of both factors in these complexes. Additional supershifted complexes (Fig. 5B, lane 2, C1) were generated relative to supershifted lysates where LEF-1 or Alx4 were expressed alone (Fig. 5A, lanes 2, C3 and C4; lane 6, C2). To determine which complexes contained Alx4 and/or LEF-1, competition with cold oligo from either the LEF-1 site (Fig. 5B, lane 3) or the P1 site (Fig. 5B, lane 4) were performed. Competition with the LEF-1 site abrogates LEF-1-DNA (C3, C4) and LEF-1/Alx4-DNA (C1) complexes, whereas competition with the P1 site abrogates Alx4-DNA (C2) and Alx4/LEF1-DNA (C1) complexes. Only Alx4 complexes remain in lane 3 (C2) and LEF-1 complexes in lane 4 (C3, C4). Both cold oligos abrogate C1 verifying the presence of both Alx4 and LEF-1 simultaneously on the N-CAM promoter oligonucleotide. Similarly, competition with the N-
CAM probe abrogates all four complexes (data not shown). Thus, we find that Alx4 binds very weakly to a P1 paired-like homeodomain consensus sequence in the N-CAM promoter region. In the presence of LEF-1, which binds to an adjacent LEF-1 site, Alx4 binding is sufficiently enhanced to detect its presence in DNA-binding complexes using EMSA. These data demonstrate the specificity of the complexes containing both Alx4 and LEF-1 observed using the wild-type N-CAM probe. Furthermore, they demonstrate that LEF-1 enhances Alx4 binding to the promoter region of N-CAM (34, 54).

Alx4 Overexpression Represses Endogenous N-CAM Expression—We next assessed the effect of Alx4 overexpression on endogenous N-CAM levels in cultures of primary mouse mammary stromal cells. We have shown previously the expression of Alx4 exclusively in the mesenchymal cells adjacent to ductal epithelia in the mouse mammary gland (35). Furthermore, the specific requirement of LEF-1 activity for the development of this tissue has also been demonstrated (15). Thus, primary mammary stromal cells were infected with a control adenovirus or a virus expressing Alx4 under the control of the CMV promoter. As Fig. 6 demonstrates, infection with the control virus had no effect on expression of the 140-kDa form of N-CAM whereas expression of Alx4 significantly reduced endogenous N-CAM levels. Thus, in agreement with the promoter

FIG. 4. Alx4 and LEF-1 regulate N-CAM promoter activity. A, schematic representation of the mouse N-CAM promoter. Nucleotides are numbered relative to the transcription start site. Filled boxes indicate putative LEF-1 and Alx4 binding sites. B, LEF-1-dependent activation of the N-CAM promoter. C3A were transiently transfected with a series of 5′-deletion N-CAM promoter constructs (50 ng) with or without Myc-tagged LEF-1 (2 μg). C, C3A were transiently transfected with the N-CAM ΔNde-CAT reporter construct, together with LEF-1 (0.5 μg) and/or LEF-1ΔHMG (0.5, 2 μg). D, Alx4 regulates LEF-1 activation of the N-CAM promoter. C3A cells were transiently transfected with the N-CAM ΔNde-CAT reporter construct, together with increasing amounts of Alx4 and/or LEF-1 expression plasmids (0.1, 0.5, 2 μg). E, Alx4 regulates LEF-1 activity by DNA binding and its interaction with LEF-1. C3A cells were transiently transfected with the N-CAM ΔNde-CAT reporter construct, together with Alx4 or Alx4ΔHMG (2 μg) and/or LEF-1 (0.5 μg) expression plasmids. Cell lysates in B, C, D, and E were assayed for CAT activity and normalized to β-galactosidase activity 48 h after transfection. Experiments were repeated five times in triplicate. Error bars indicate the standard error of the mean.
assays in which high levels of Alx4 (relative to LEF-1) inhibited transcription from the N-CAM promoter, adenovirus-mediated expression of Alx4 can repress expression of endogenous N-CAM protein in primary mouse mammary stromal cells.

DISCUSSION

While binding to and altering the structure of DNA (34, 54), LEF-1 alters gene transcription through direct association with additional transcription factors (14, 18–29). By virtue of many distinct protein-binding domains throughout the protein, LEF-1 integrates signals from a number of distinct developmental pathways. For the development of hair, whiskers, teeth, and breast tissue, integration of these signals by LEF-1 is an essential requirement for development (15).

Homeobox genes have been shown to regulate genes encoding several cell adhesion molecules that mediate interactions leading to morphogenesis (55). Specifically, the neural cell adhesion molecule (N-CAM) contains homeodomain binding sites that are composed of a conserved ATTA core motif but vary with respect to base pairs flanking this motif. The sequence composition of the homeodomain binding sites determines which homeodomain proteins can interact with these regulatory sequences. The present study identifies Alx4 as a potential modifier of N-CAM expression both in promoter assays and cultures of primary mammary stromal cells. In order for Alx4 to repress the N-CAM promoter it must affect the function of activators, specifically LEF-1, bound to other sites within the promoter.

Because in developing hair follicles, expression of LEF-1 occurs in both mesenchymal and epithelial compartments, differences in LEF-1-mediated expression of genes would be expected to occur through the binding of factors that are expressed uniquely in these distinct compartments. Alx4 is an example of a factor whose expression is restricted to condensing mesenchymal cells adjacent to the invaginating epithelia in the developing embryonic hair follicle. Alx4, like LEF-1, has an

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**FIG. 5.** 
A, Alx4 and LEF-1 bind the N-CAM promoter. Electrophoretic mobility shift assays were performed using an N-CAM promoter fragment containing the upstream LEF-1 binding site and the P1 site. COS-1 nuclear lysates containing LEF-1 or Alx4 were incubated with radiolabeled double-stranded N-CAM probe. Supershifts were performed using α-Myc or α-Alx4 monoclonal antibodies (lanes 2 and 3 and lanes 6 and 7, respectively). Competition with a 200-fold cold N-CAM probe was performed (lanes 3, 4, 7, 8, 11, 13, and 14). B, Alx4 and LEF-1 bind the N-CAM promoter simultaneously. COS-1 nuclear lysates containing both LEF-1 and Alx4 were incubated with radiolabeled double-stranded N-CAM probe. Supershifts were performed using α-Myc and α-Alx4 monoclonal antibodies (lanes 2–4). Competition with either 200-fold cold LEF-1 site oligo (lane 3) or cold P1 site oligo (lane 4) was performed.

**FIG. 6.** 
A, Alx4 and LEF-1 bind the N-CAM promoter. Electrophoretic mobility shift assays were performed using an N-CAM promoter fragment containing the upstream LEF-1 binding site and the P1 site. COS-1 nuclear lysates containing LEF-1 or Alx4 were incubated with radiolabeled double-stranded N-CAM probe. Supershifts were performed using α-Myc or α-Alx4 monoclonal antibodies (lanes 2 and 3 and lanes 6 and 7, respectively). Competition with a 200-fold cold N-CAM probe was performed (lanes 3, 4, 7, 8, 11, 13, and 14). B, Alx4 and LEF-1 bind the N-CAM promoter simultaneously. COS-1 nuclear lysates containing both LEF-1 and Alx4 were incubated with radiolabeled double-stranded N-CAM probe. Supershifts were performed using α-Myc and α-Alx4 monoclonal antibodies (lanes 2–4). Competition with either 200-fold cold LEF-1 site oligo (lane 3) or cold P1 site oligo (lane 4) was performed.

C, complex.

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important role in the development of a number of tissues including hair follicles (36, 38). We also have observed Alx4 expression exclusively in mesenchymal cells in a number of tissues whose development is influenced by LEF-1 activity (37). In the context of hair follicle development, the absence of Alx4 in mice results in a significant decrease in dermal thickness, a reduction in the number of melanocytes, little or no adipose tissue development, and a decrease in the size of the dermal papilla (38). Thus, based on the overlapping expression (37), primary amino acid sequence, and defects that arise during hair follicle development, we hypothesized that Alx4 might impart some mesenchymal cell-specific activities to LEF-1. In agreement with our hypothesis, we have demonstrated that the interaction between these two factors alters the promoter activity and endogenous levels of the cell adhesion molecule, N-CAM. Regulation of the N-CAM promoter appears to be mediated through adjacent but distinct LEF-1 and Alx4 binding sites in the N-CAM promoter. Our results indicate that the effects of Alx4 on LEF-1 activity are dependent on levels of Alx4 and LEF-1 expression. In addition, the promoter assays demonstrate that the Alx4 mutant, which is unable to bind DNA but can bind LEF-1, has no effect on LEF-1 activity, suggesting that DNA binding of Alx4 is required for Alx4 repressor activity. However, as demonstrated in EMSAs, LEF-1 enhances Alx4 binding to DNA, suggesting that both interaction with LEF-1 and DNA binding are required to mediate its effects on the N-CAM promoter.

As shown in the EMSAs, nuclear extracts with LEF-1 and Alx4 show that both specifically bind to the N-CAM promoter. In addition, complexes containing both factors are also apparent, demonstrating that both Alx4 and LEF-1 bind simultaneously to the N-CAM promoter. The relatively lower levels of LEF-1/Alx4 co-complexes may reflect the weak binding of Alx4 for paired-like homeodomain binding sites observed in EMSAs (36, 53, 56). In fact, our data suggest that the presence of LEF-1 influences Alx4 DNA binding activity because weak complexes containing Alx4 are seen in the presence of LEF-1 (see arrows in Fig. 5B, lane 1) but are absent when LEF-1 is not present (Fig. 5A, lane 5). Homeodomain proteins can interact with DNA as monomers and recruit other factors to these sites (52, 58). Previous studies have demonstrated that some paired-like homeodomain proteins can also mediate transcriptional effects through P1/2 sites. For example, Phox1 binding to a P1/2 site is enhanced by interactions with serum response factor, which binds an adjacent site (59). Likewise, Alx4 acts on half-sites that are juxtaposed to target sites of other DNA binding partners (53). It is clear, however, that additional developmental signals also impinge on Alx4 because the effects seen for Alx4 mutant mice are evident primarily on the dorsal side of the animals, the ventral having a greater resemblance to wild-type animals (35). This dorsal effect is not caused by differences in the apparent expression patterns of Alx4 in the dorsal-ventral axis. Rather, the defect appears to be due to the influence of a dorsal signal whose integration requires the presence of Alx4. Presumably, this signal is genetically upstream of LEF-1 because no hair follicle development occurs in its absence.

The expression of N-CAM has been observed in a variety of sites of mesenchymal morphogenesis, including feather and hair dermal condensations and precartilaginous condensations (48, 57, 60, 61). In these cases, there are sites where both Alx4 and LEF-1 expression intersect. N-CAM is expressed in the hair placode and mesenchyme during hair follicle development (48) and is important for the formation of dermal condensation during feather bud morphogenesis (60). When N-CAM is blocked via antibodies, dermal condensations required for feather condensations do not form. In mice deficient in LEF-1, these condensations also fail to form, as do hair follicles. Consistent with our observation that expression of exogenous LEF-1 causes transactivation of the N-CAM-promoter, a recent study demonstrated that noggin-dependent induction of LEF-1 coincidentally increased N-CAM expression (50). Ectopic noggin added to skin cultures up-regulates LEF-1 expression and stimulates hair induction. Based on promoter assays and EMSAs, our results further support the notion that N-CAM is a direct target of LEF-1. The data presented here, in conjunction with studies of the various mutant mouse models, suggest that Alx4 binding to LEF-1 generates a complex that can regulate promoter activity. Our results reveal a further complexity in regulation by Alx4 and LEF-1 of target genes. Specifically, the stoichiometry between these factors appears to determine whether activation or repression of promoter activity occurs. Because the levels of Alx4 appear to change in specific tissues over the course of time, the regulation of target genes by Alx4 and LEF-1 may in fact be very dynamic. We expect to address this hypothesis employing Alx4-mutant heterozygous and homozygous animals.

In conclusion, we have demonstrated a directed and functional interaction between the HMG-box transcription factor (LEF-1) and the paired-like homeodomain protein (Alx4). These two factors bind simultaneously to adjacent sites in the N-CAM promoter and modulate its transcriptional activity.

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