

Scleraxis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis

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SUMMARY

Members of the basic helix-loop-helix (bHLH) family of transcription factors have been shown to regulate growth and differentiation of numerous cell types. Cell-type-specific bHLH proteins typically form heterodimers with ubiquitous bHLH proteins, such as E12, and bind a DNA consensus sequence known as an E-box. We used the yeast two-hybrid system to screen mouse embryo cDNA libraries for cDNAs encoding novel cell-type-specific bHLH proteins that dimerize with E12. One of the cDNAs isolated encoded a novel bHLH protein, called scleraxis. During mouse embryogenesis, scleraxis transcripts were first detected between day 9.5 and 10.5 post coitum (p.c.) in the sclerotome of the somites and in mesenchymal cells in the body wall and limb buds. Subsequently, scleraxis was expressed at high levels within mesenchymal precursors of the axial and appendicular skeleton and in cranial mesenchyme in

advance of chondrogenesis; its expression pattern in these cell types foreshadowed the developing skeleton. Prior to formation of the embryonic cartilaginous skeleton, scleraxis expression declined to low levels. As development proceeded, high levels of scleraxis expression became restricted to regions where cartilage and connective tissue formation take place. Scleraxis bound the E-box consensus sequence as a heterodimer with E12 and activated transcription of a reporter gene linked to its DNA-binding site. The expression pattern, DNA-binding properties and transcriptional activity of scleraxis suggest that it is a regulator of gene expression within mesenchymal cell lineages that give rise to cartilage and connective tissue.

Key words: bHLH protein, sclerotome, somites, chondrogenesis, mouse, scleraxis, skeletal formation

INTRODUCTION

Members of the basic helix-loop-helix (bHLH) family of transcription factors have been shown to play important roles in the control of cell differentiation, proliferation and oncogenesis (for review, see Kadesch 1993). The bHLH motif is composed of a basic region followed by two predicted amphipathic α -helices separated by an unstructured loop (Murre et al., 1989a). The HLH region mediates dimerization and brings together the basic regions of bHLH proteins to form a bipartite DNA-binding domain that binds a subset of sequences sharing the consensus CANNTG, known as an E-box (Murre et al., 1989a,b).

There are several subclasses of bHLH proteins, distinguishable by their dimerization specificities, DNA-binding preferences and expression patterns. Class A bHLH proteins are widely expressed and dimerize preferentially with Class B bHLH proteins, which are cell-type-specific (Murre et al., 1989a,b). Examples of Class A bHLH proteins are the E2A gene products E12 and E47 (Murre et al., 1989a), HEB (Hu et al., 1992), and the *Drosophila daughterless* gene product

(Caudy et al., 1988). Among the most well characterized Class B bHLH proteins are the myogenic regulatory factors MyoD, myogenin, myf5 and MRF4; the *Drosophila* and mammalian *achaete scute* gene products, which regulate neurogenesis; and twist, which controls mesoderm formation (reviewed in Jan and Jan, 1993). Although Class A and B bHLH proteins can homodimerize, heterodimerization is preferred and is likely to be responsible for the biological activity of these proteins in vivo (Murre et al., 1989a; Davis et al., 1991; Lassar et al., 1991; Chakraborty et al., 1991).

The widespread expression of Class A bHLH proteins and the presence of functional E-boxes in the control regions of numerous cell-type-specific genes suggest the existence of cell-specific bHLH proteins in many cell types. Although members of the bHLH family share conserved amino acids within the bHLH region, the extent of nucleotide homology among the genes encoding these proteins is in most cases insufficient to allow cross-hybridization between divergent family members. Therefore, to search for novel cell-type-specific bHLH proteins, we sought to exploit the ability of cell-specific bHLH proteins to dimerize selectively with ubiquitous Class

A bHLH proteins. Using the yeast two-hybrid system (Fields and Song, 1989), we screened a mouse embryo cDNA expression library for cDNAs whose products could dimerize with E12. Here we describe the isolation of a cDNA encoding a novel bHLH protein, referred to as scleraxis, which was first expressed in the sclerotomal compartment of the somites and subsequently appeared in mesenchymal primordia of bones and cartilage, and in tendons and ligaments. Scleraxis dimerized and bound the E-box consensus sequence with E12 and activated transcription throughout its multimerized DNA-binding site in transfected cells. Its expression pattern, DNA-binding properties and transcriptional activity suggest that scleraxis may regulate gene expression within chondrogenic lineages during embryogenesis.

MATERIALS AND METHODS

Yeast expression vectors

The GAL4(DBD)-E12bHLH fusion vector was constructed by inserting the region encoding amino acids 502-648 of E12 (Murre et al., 1989a) into the *Sma*I site of the yeast expression plasmid pPC62 (Chevray and Nathans, 1992). This generates a chimeric cDNA in which amino acids 1-147 of yeast GAL4, which encompass the DNA-binding domain (DBD), are in-frame with E12. The region of E12 introduced into this vector encompasses the bHLH region, but it lacks the transcription activation domain. Expression and stability of the resulting GAL4(DBD)-E12 fusion protein was confirmed by western blot analysis (data not shown). A GAL4(TAD) fusion expression library, constructed from cDNA from day 14.5 post coitum (p.c.) mouse embryonic poly(A)⁺ RNA, was generously provided by P. Chevray (Johns Hopkins Medical School, Baltimore, MD) (Chevray and Nathans, 1992). This library contains cDNAs fused to the transcription activation domain (TAD) of GAL4.

Yeast strains and methods

Yeast strain PCY2 (MAT α Δ gal4 Δ gal80 URA::3GAL1-lacZ lys2-801^{amber} his3- Δ 200 trp1- Δ 63 leu2 ade2-101^{ochre}) (Chevray and Nathans, 1992) was used in an initial screening of $\sim 5 \times 10^5$ colonies. Yeast transformed with GAL4(DBD)-E12 bHLH were grown under Leu selection, transformed with 10 μ g of embryonic library DNA, and plated on SD-Trp-Leu plates.

Colonies with reconstituted GAL4 activity were identified by assaying for β -galactosidase as described previously (Staudinger et al., 1993). Plasmids from β -galactosidase positive colonies were isolated and transformed into *Escherichia coli* ElectroMAX DH10B cells (GIBCO/BRL).

DNA sequencing and analysis

cDNA inserts were excised from the GAL4(TAD) plasmids by digestion with *Not*I plus *Sal*I and subcloned into pBSKII+ (Stratagene). Both strands of plasmids were sequenced using a combination of nested deletions and oligonucleotide primers in conjunction with dideoxy sequencing using Sequenase (United States Biochemical) according to manufacturer's instructions. Sequence analysis was carried out using the GCG sequence analysis software package.

To obtain full-length scleraxis cDNAs, we screened a day 13 p.c. mouse embryo cDNA library (Novagen) at high stringency with a 400-bp fragment from the 5' end of the scleraxis cDNA obtained from the yeast screen.

In situ hybridization

Sense and anti-sense ³⁵S-labeled probes were synthesized from a 1.2-kb scleraxis cDNA insert cloned into pBSKII+ using T3 and T7 RNA polymerases, respectively. The *Pax-1* probe used for in situ

hybridization was described by Deutsch et al. (1988). In situ protocols were as previously described (Edmondson et al., 1994).

In vitro transcription and translation and DNA-binding assays

A glutathione-S-transferase (GST)-E12 fusion protein was created by cloning a portion of the E12 cDNA encompassing amino acids 210-648, which includes the bHLH region, in-frame with GST (Chakraborty et al., 1991). A polyhistidine-scleraxis fusion protein was created by cloning a portion of the scleraxis cDNA encompassing amino acids 9-207 in-frame with the metal-binding domain of pRSET (Invitrogen). GST-E12 was purified from bacterial extracts using glutathione-agarose beads as described (Chakraborty et al., 1991). Polyhistidine-scleraxis was purified by metal affinity chromatography according to manufacturer's instructions (Invitrogen).

In vitro transcription and translation of scleraxis and E12 was performed using the TnT kit (Promega) according to manufacturer's instructions. Following translation, 5 μ l of lysate was used in gel mobility shift assays. Parallel translations were performed in the presence of [³⁵S]methionine to ensure that the proteins were synthesized.

A double-stranded oligonucleotide probe corresponding to the left E-box from the mouse muscle creatine kinase (MCK) enhancer (Sternberg et al., 1988; Lassar et al., 1989) was labeled with ³²P and used as a probe for gel mobility shift assays. The sequence of the probe was CCCAGACATGTGGCTGCTCCC. For competition experiments, a 100-fold molar excess of unlabeled oligonucleotide was included in the binding reaction. DNA-binding assays were performed as described (Chakraborty et al., 1991) using aliquots of in vitro translated proteins or 500 ng of the indicated bacterial fusion proteins.

Transfection assays

To test the transcriptional activity of scleraxis, four tandem copies of an E-box with the following sequence (CCGAACACATGTGCC-CCGC) were inserted upstream of the thymidine kinase gene promoter and the chloramphenicol acetyltransferase (CAT) reporter. This E-box binds scleraxis/E12 heterodimers with high affinity (D. B., unpublished results). The scleraxis cDNA was cloned into the vector pECE-Flag (Ellis et al., 1986) such that the 9 amino acid recognition sequence for hemagglutinin monoclonal antibody was fused in-frame to amino acid 8 of scleraxis. This expression vector contains the SV40 promoter and enhancer. The 3' untranslated region of the mouse protamine gene was inserted downstream of the scleraxis cDNA.

COS-1 cells were transiently transfected as described previously (Sternberg et al., 1987) with 5 μ g of tk-CAT or 4E-tkCAT and 5 μ g of scleraxis expression vector, empty expression vector, or EMSV-MyoD (Davis et al., 1987). 48 hours after transfection, cells were harvested and CAT activity was determined in cell extracts.

Interspecific mouse backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J \times *M. spretus*)F₁ females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N₂ mice were used to map the *Scx* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described (Jenkins et al., 1982). All blots were prepared with Hybond-N⁺ nylon membrane (Amersham). The probe, an ~ 600 bp *Stu*I/*Not*I fragment of mouse scleraxis, cDNA was labeled with [α -³²P]dCTP using a random primed labeling kit (Stratagene); washing was done to a final stringency of 0.8 \times SSCP, 0.1% SDS, 65°C. Fragments of 10.1 and 2.5 kb were detected in *Eco*RI-digested C57BL/6J DNA, and fragments of 6.0 and 2.5 kb were detected in *Eco*RI-digested *M. spretus* DNA. The presence or absence of the 6.0 kb *M. spretus*-specific *Eco*RI fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Scx* including thyroglobulin (*Tgn*), platelet-derived growth factor beta (*Pdgfb*) and wingless related MMTV integration site 1 (*Wnt1*) has been given previously (Brannan et al., 1992). Recombination distances were calculated as described (Green, 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS

Screening for E12 dimerization partners using the yeast two-hybrid system

To search for novel bHLH proteins that dimerize with E12, we used the yeast two-hybrid system (Fields and Song, 1989), which relies on protein-protein interactions to reconstitute a functional transcription factor from two separate proteins, one of which carries a DNA-binding domain (DBD), the other a transcription activation domain (TAD). For this screen, a plasmid was created in which the bHLH region of E12 was fused to the DBD of yeast GAL4. We showed previously that this GAL4-E12 fusion protein fails to activate the expression of marker genes under control of the GAL4 DNA-binding site in yeast. However, when the GAL4-E12 fusion protein was coexpressed with a plasmid encoding the bHLH region of MyoD fused to the GAL4 TAD, GAL4-dependent marker genes were activated, as a consequence of dimerization of E12 and MyoD (Staudinger et al., 1993; see also Chakraborty et al., 1992). Thus, it appeared that the GAL4-E12 fusion protein would provide a sensitive and specific means of identifying cDNAs encoding novel dimerization partners for E12 from cDNA expression libraries in yeast.

To screen for E12 dimerization partners, we transformed a yeast strain harboring a GAL4-dependent *lacZ* reporter gene with the GAL4-E12 expression vector and a cDNA library from day 14.5 p.c. mouse embryos in which cDNAs were fused to the GAL4-TAD (see Materials and Methods). Screening of $\sim 5 \times 10^5$ independent transformants yielded three cDNAs capable of activating *lacZ* expression. Specificity of the interactions between E12 and the proteins encoded by the positive cDNA clones was tested by rescuing the activating plasmids from yeast and retransforming them into the same yeast strain alone or with expression plasmids encoding unrelated proteins fused to the GAL4 DBD. None of the activating plasmids were able to activate the *lacZ* marker gene in the absence of GAL4-E12. Based on the time required to induce *lacZ* activity and the intensity of *lacZ* staining, the activating clones appeared to be more potent than the MyoD-activating plasmid (Staudinger et al., 1993) in inducing expression of the *lacZ* marker gene.

The nucleotide sequences and deduced open reading frames of the activating cDNAs were determined by DNA sequencing. Two of the cDNAs were overlapping and the other was distinct. A search of the sequences against the database showed that the cDNAs encoded novel bHLH proteins. We chose to focus on the two cDNAs that were overlapping. The protein encoded by these cDNAs was called scleraxis, because it was first expressed in the sclerotome and in progenitors of the axial skeleton during mouse embryogenesis (see below). The properties of the other novel bHLH protein will be described elsewhere (P. Cserjesi and E. Olson, unpublished data).

Because the yeast two-hybrid system selects for fusion proteins in which the cloned protein is fused in-frame to amino acid 147 of yeast GAL4, the rescued cDNAs are truncated within the protein coding region. We therefore used the original scleraxis cDNA to rescreen an embryo cDNA library for cDNAs encompassing the complete open reading frame. The composite nucleotide sequence obtained from several overlapping scleraxis cDNAs was 1140 bp in length and contained a polyadenylation signal followed by a poly(A) tail at its 3' end (Fig. 1A). The size of the cloned cDNA corresponds to that of the scleraxis transcript detected by northern blot (data not shown), suggesting that the cDNA encompasses most or all of the transcript. The cDNA sequence predicts that scleraxis is a 207-amino acid protein with a $M_r=22,238$ ($pI=10.9$), which is slightly smaller than the size of the in vitro translation product obtained from the scleraxis mRNA (see below).

The homology between scleraxis and other bHLH proteins is shown in Fig. 1C. Within the bHLH region, scleraxis showed 61% identity to mouse twist (Wolf et al., 1991). The hematopoietic bHLH proteins NSCL-1 (Begley et al., 1992), *lyl-1* (Mellentin et al., 1989) and *tal-2* (Xia et al., 1991) also showed greater than 50% identity to scleraxis within the bHLH region. Notably, all of the conserved amino acids that define the bHLH family of transcription factors were conserved in scleraxis, including the basic amino acids that have been shown to mediate DNA binding of other bHLH proteins (Davis et al., 1991; Brennan et al., 1991). Other residues in the basic region are divergent, suggesting that scleraxis may bind a subset of E-boxes distinct from that of other bHLH proteins. Scleraxis also lacks the residues in the basic region that have been shown to mediate muscle gene activation by myogenic bHLH factors (Davis et al., 1991; Brennan et al., 1991).

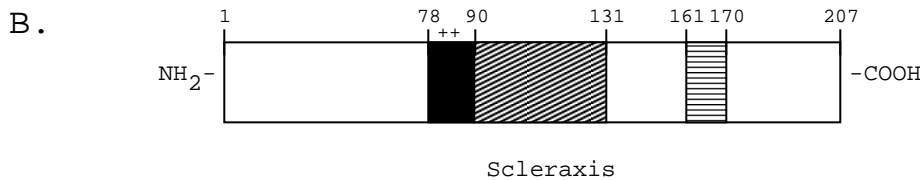
Outside the bHLH region, scleraxis showed no significant homology to other known bHLH proteins. Other notable features of scleraxis include a proline-rich region near the carboxyl-terminus (Fig. 1A,B) similar to transcription activation domains in several transactivator proteins (Mermod et al., 1989). There are consensus sites for phosphorylation by casein kinase II (residues 24 and 34), protein kinase A (residues 115 and 206), and protein kinase C (residue 119) (Kemp and Pearson, 1990).

Scleraxis transcripts are expressed in the sclerotome, the developing skeleton, and connective tissues

The expression pattern of scleraxis transcripts during mouse development was determined by in situ hybridization to embryos that were 7.5 through 16.5 days p.c. We detected very low basal levels of scleraxis transcripts throughout development. However, the first significant expression that was localized to specific cell types was observed between days 9.5 and 10.5 p.c. in the lateral sclerotome and in mesenchymal cells of the limb buds and body wall (Fig. 2B). Scleraxis transcripts were not detected in the somites prior to compartmentalization nor were they present at significant levels in the myotomes or dermatomes of the somites.

The paired box gene *Pax-1* is expressed in a portion of the sclerotome beginning at about day 9 p.c. (Deutsch et al., 1988) and has been implicated in sclerotome differentiation (Wallin et al., 1994). To determine whether *Pax-1* and scleraxis define the same regions of the sclerotome, we compared the

A.
 CTCGGCTGGCGGCCCACTCCAGTCCGAACACATGTGCCCGCCTACCCGGCAGCCGCCA 60
 CCGCGGGGCGCAGCGGAGACCCCTGGCCCGGGCGGCTGTGGGGACCTAAAGAGGGCGGCAT 120
 GAGCAGCGCACGGTGGAGCTGACGCCGCGCCCCCTGCCCGGCCCATGTCTTCGCCATGC 180
 M S F A M L 6
 TGCCTTCAGCGCCGCCCGGGTTCGTACCTGTACCCTGAGGTGAGCCCGCTGTCCGGAGG 240
 R S A P P P G R Y L Y P E V S P L S E D 26
 ATGAGGACCCGGAAGCGAGAGCTCGGGCTCCGACGAGAAACCTGCCGTGTGCATGCTG 300
 E D R G S E S S G S D E K P C R V H A A 46
 CGCGCTGTGGCTCCAGGGCGCCCGGGCGGGCAGGAGGACGGAGGGCCGCGGGTAGCG 360
 R C G L Q G A R R R A G G R R A A G S G 66
 GGCCAGACCCGGGGGGCGCCAGGCCGCGAGCCCCGGCAGCGGCACACAGCGAATGCGC 420
 P G P G G R P G R E P R Q R H T A N A R 86
 GCGAGCGGGACCGCACCAACAGCGTGAACACGGCCTTCACTGCGCTGCGCACACTCATCC 480
E R D R T N S V N T A F T A L R T L I P 106
 CCACCGAGCCAGCGGACCGCAAGCTTCCAAGATTGAGACGCTGCGCCTGGCCTCCAGCT 540
T E P A D R K L S K I E T L R L A S S Y 126
 ACATTTCTACCTGGCAATGTGCTGCTGGTGGGTGAGCCTGTGGCGACGGGCAACCAT 600
I S H L G N V L L V G E A C G D G Q P C 146
 GCCACTCGGGACCCGCTTTCTTCCACAGCGGTGCTGCGGGCAGCCCACTGCCACCGCCGC 660
 H S G P A F F H S G R A G S P L P P P P 166
 CGCCGCCACCACCTGGCCAGAGACGGCGGCGAGAACACCCAGCCCAAACAGATCTGCA 720
 P P P P L A R D G G E N T Q P K Q I C T 186
 CCTTCTGCCTCAGCAACCAGAGAAAGTTGAGCAAAGACCGTGACAGAAAGACGGCGATTC 780
 F C L S N Q R K L S K D R D R K T A I R 206
 GAAGTTAGAAGGAGGGTCCACAGCAGCCAAGAGGTGATGCCACTAGTGAAGGTGGAT 840
 S * 207
 GCCACTGAAGAGTACGGAGAGCCCCACTTTGGACCTGATCTGAGCGCAGGTCCCCAAGA 900
 GCACGCTCACCAGGCCAGACCTGCCCCAGCTATGAGCAGGGCCTATGAACAGAGAGATGG 960
 ACAGGCGGGTGATGGCTTGACTCTAAGCAGCCGAACACTGACCCAGGCCCACTGGGACTT 1020
 TCCATGCTGGCTCTTACCTGGGCCAAGTGTGGCATCTTGTGTCTTTAATATGATAATA 1080
 TAAAGTCTGAAATTTGTATAAATTAATAAATGAAACAGTATCTTCCAAAAAAAAAAAAA 1140



C.
 ←Basic→|←Helix-1→|←Loop→|←Helix-2→
 scleraxis RQRHTANARERDRTNSVNTAFTALRTLIPTEPADRKLKSKIETLRLLASSYISHLG
 mtwist T--VM--V---Q--Q-L-E-RA---KI---L-S-.-----Q--K--AR--DF-Y
 NSCL-1 KY-TAHAT---I-VEAF-L--AE--K-L--L-P-K-----I----IC---Y-N
 lyl-1 AR-VFT-S---W-QQN--G--AE--K-L--H-P-K-----N-V---MK--GF-V
 tal-2 TRKIIFT-T---W-QQN--S--AK--K-L--H-P-K-----N-----MR--NF-V
 Myogenin DR-KA-TM---R-LSK--E--ET-KRCTSSN-NQ-. -P-V-I--N-IR--EG-Q
 MASH-1 AR-...-E---N-VKL--LG-AT--EHV-NGA-NK-M--V---S-VE--RA-Q
 consensus R N ER R ψ F L ψ Kψ IL Aψ Yψ L V
 T T L V V

Fig. 1. Nucleotide sequence of scleraxis cDNA and deduced open reading frame of scleraxis protein. (A) Nucleotide sequence and predicted open reading frame of scleraxis. The bHLH region, indicated in bold, encompasses residues 78-131. A proline-rich region lies between residues 161-170. (B) Schematic of scleraxis protein. ++, basic region. (C) Homologies between the bHLH region of HLH2 and other bHLH proteins. A dash indicates amino acid identity at that position. Sources of sequences are Wolf et al. (1991), mouse twist; Begley et al. (1992), human NSCL-1; Mellentin et al. (1989), human lyl-1; Xia et al. (1991), human tal-2; Edmondson and Olson (1989), mouse myogenin; Johnson et al. (1990), rat MASH-1.

expression patterns of the two genes by in situ hybridization to sequential sections of embryos at day 10.5 p.c. Scleraxis expression at this stage was highest in the ventrolateral region of the sclerotome, whereas *Pax-1* expression was concentrated in the ventromedial region between the notochord and the neural tube (Fig. 2B and C), as described previously (Deutsch et al., 1988; Wallin et al., 1994). Whereas scleraxis was

expressed initially in the lateral region of the sclerotome, scleraxis expression eventually extended into the ventromedial region of the sclerotome (Fig. 3A). Thus, scleraxis and *Pax-1* show distinct temporal and spatial expression patterns and demarcate different regions of the sclerotome.

Parasagittal sections showed high levels of scleraxis expression in a metameric pattern extending along the length

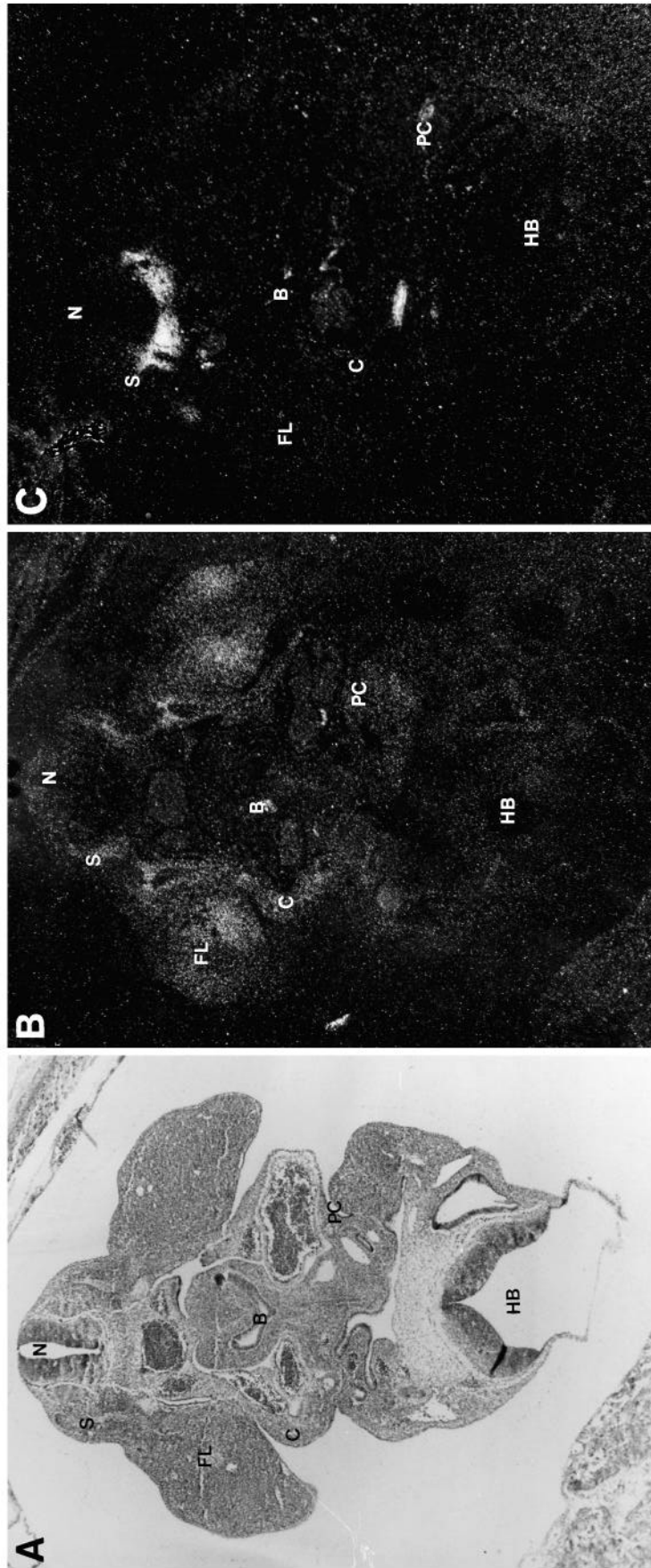


Fig. 2. Expression of scleraxis and *Pax-1* transcripts in different domains of the sclerotome. Transcripts for scleraxis and *Pax-1* were detected by in situ hybridization to sequential transverse sections of a day 10.5 p.c. mouse embryo at the level of the forelimb. (A) Bright-field, (B) anti-sense scleraxis probe, (C) anti-sense *Pax-1* probe. B, lung buds; C, chest wall; FL, forelimb; HB, hindbrain; N, neural tube; PC, pharyngeal cleft; S, sclerotome.

of the rostrocaudal axis of the embryo at day 11.0 p.c. (Fig. 4A). At the rostral end of the embryo, these condensations of scleraxis expression represent the precursors of the vertebrae and the intervertebral discs. Scleraxis expression in the sclerotomes of the less mature caudal somites could also be observed at this stage. Scleraxis transcripts were also expressed in the rib primordia, as well as within mesenchymal cells in the limb buds and frontonasal region. Mesenchymal cell masses at the base of the tongue, which give rise to the thyroid cartilage and the hyoid bone, also showed high levels of scleraxis expression. The pericardium also contained scleraxis-expressing cells (Fig. 4A).

By day 11.5 p.c., expression of scleraxis in the developing vertebrae and intervertebral discs had extended caudally (Fig. 4B). Frontal sections of day 11.5 p.c. embryos revealed high expression of scleraxis transcripts in precursors of the ribs and bones of the limbs (Fig. 5B). Its expression in progenitors of the axial and appendicular skeleton preceded ossification of the bones by at least 2 days. Cross-sections through the future ribs and long bones showed that scleraxis expression was confined to the chondroblasts and chondroblast precursors at the periphery, with no expression in mature chondrocytes in the central core of these presumptive bones (Fig. 5B).

Scleraxis transcripts were not detected in precursors of the calvarial bones, but were present at high levels in mesenchymal cells in the frontonasal region. Transverse sections through the somites also showed scleraxis expression in the lateral regions of the rostral sclerotomes at day 11.5 p.c. (Fig. 5C). The highly localized expression of scleraxis in the somites was also revealed by tangential sections through the caudal region of a day 12.5 p.c. embryo (Fig. 3B). Scleraxis transcripts were also present in the sternal region of the chest wall, as well as in the diaphragm, tongue and heart valves (Figs 4A-C, 5C), all of which contain connective tissue. We cannot state with certainty that scleraxis is expressed in connective tissue within the muscular tissues of the tongue and diaphragm, but we believe this to be the case based on its restriction to connective tissue and chondrogenic cells.

Within 48 hours after the rib anlagen were first detected, scleraxis expression declined to basal levels (Fig. 4B,C). Expression in the anlagen of the long bones became restricted to the less mature regions of the embryo, such as the pelvic region and epiphyseal plates of the developing limbs. Concurrently, expression in regions of future dense connective tissue such as the diaphragm, bronchi of the lungs, heart valves and ligaments of the spine increased dramatically. By day 14.5 p.c. (Fig. 5D), the high levels of scleraxis expression

were restricted predominantly to regions that develop into dense connective tissues such as tendons, ligaments and cartilage. Within the developing face and skull, scleraxis was expressed in the facial region during early development, but it was never detected in regions of intramembranous bone formation in the cranium. Scleraxis was also not expressed in the irregular dense connective tissue of the skin.

While scleraxis expression was clearly concentrated in developing connective tissue and cartilage, very low levels of scleraxis expression could be detected in a wide range of adult tissues by northern analysis (data not shown). Since many tissues contain a connective component, scleraxis expression may reflect the presence of connective cells.

Scleraxis binds DNA as a heterodimer with E12

The presence of conserved basic amino acids in the basic region of scleraxis suggested that it had the potential to bind DNA. To test for DNA-binding activity, we expressed scleraxis as a histidine (His) fusion protein and looked for its possible binding to a series of labeled probes corresponding to different E-box sequences. The His-scleraxis fusion protein showed little specific DNA-binding activity alone, whereas in the presence of a GST-E12 fusion protein, significant binding to a sequence corresponding to the left E-box from the MCK enhancer was detected (Fig. 6A). DNA binding by His-

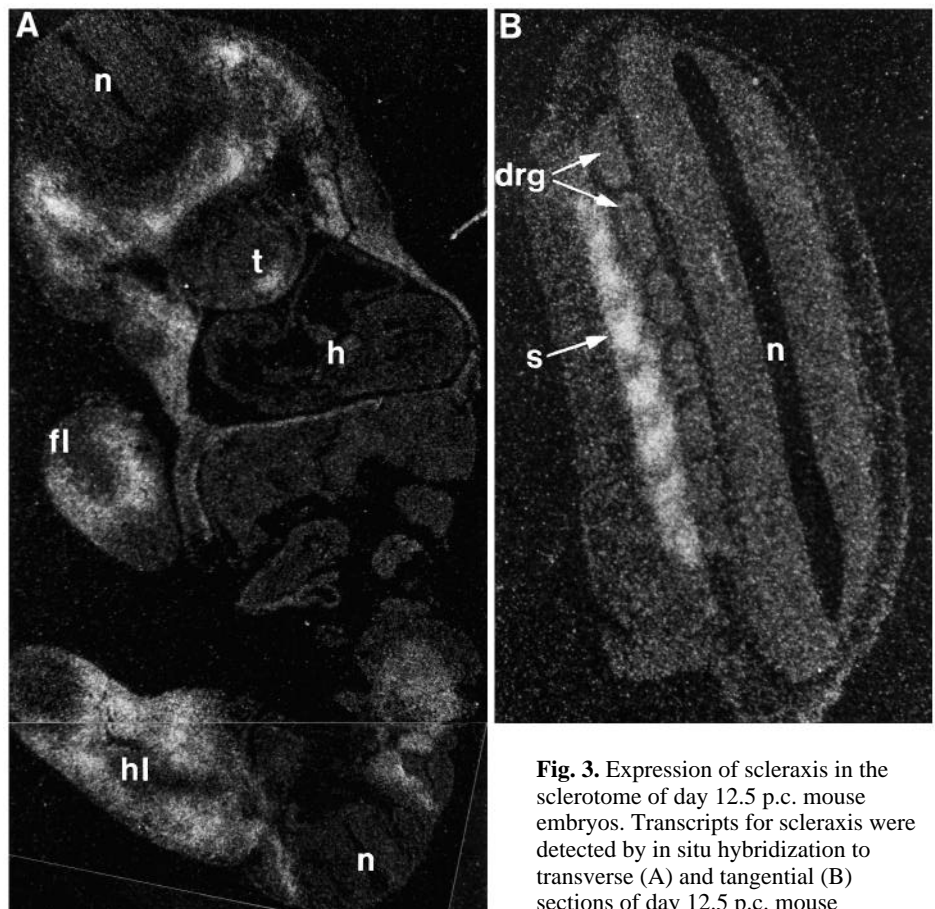


Fig. 3. Expression of scleraxis in the sclerotome of day 12.5 p.c. mouse embryos. Transcripts for scleraxis were detected by in situ hybridization to transverse (A) and tangential (B) sections of day 12.5 p.c. mouse embryos. In A, scleraxis transcripts can

be seen throughout the sclerotome and limb buds, as well as in the body wall and trachea. The expression of scleraxis in the somites can also be seen in B. drg, dorsal root ganglia; fl, forelimb bud; h, heart; hl, hindlimb bud; n, neural tube; s, sclerotome; t, trachea.

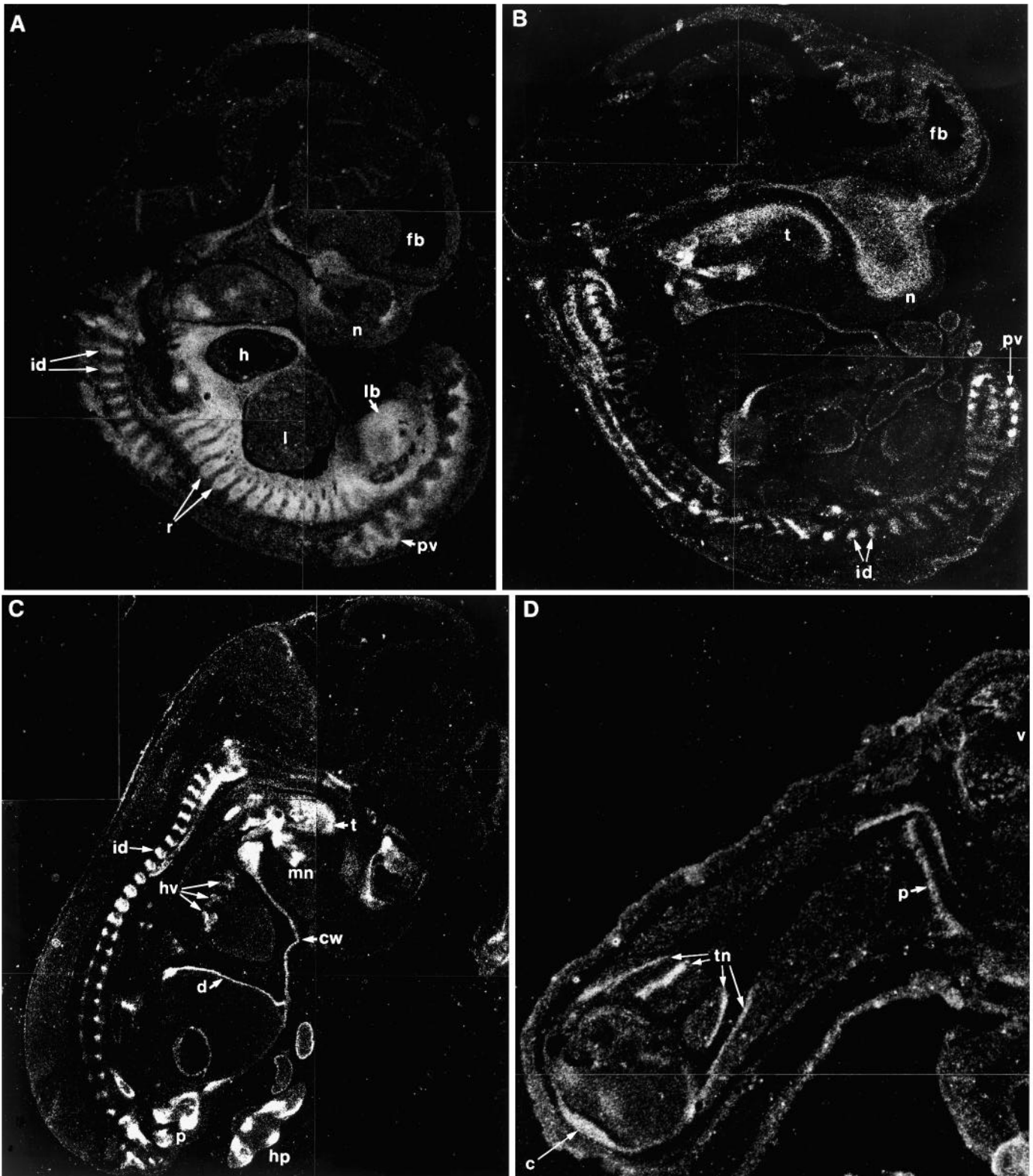


Fig. 4. Detection of scleraxis transcripts on sequential days of mouse development by in situ hybridization. Transcripts for scleraxis were detected by in situ hybridization to parasagittal sections of (A) day 11 p.c., (B) day 11.5 p.c., and (C) day 12.5 p.c. mouse embryos. Sense probes for scleraxis showed no detectable hybridization above background. (D) A transverse section through the pelvis and hindlimb of a day 15.5 p.c. embryo. Scleraxis transcripts are present in the intervertebral discs (id), prevertebrae (pv), pre-ribs (r), condensing cartilage of the nose and face, and in connective tissues. c, cartilage; cw, chest wall; d, diaphragm; fb, forebrain; h, heart; hp, hindpaw; hv, heart valves; l, liver; lb, limb bud; mn, mandible; n, nose; p, pelvis; tn, tendon; t, tongue.

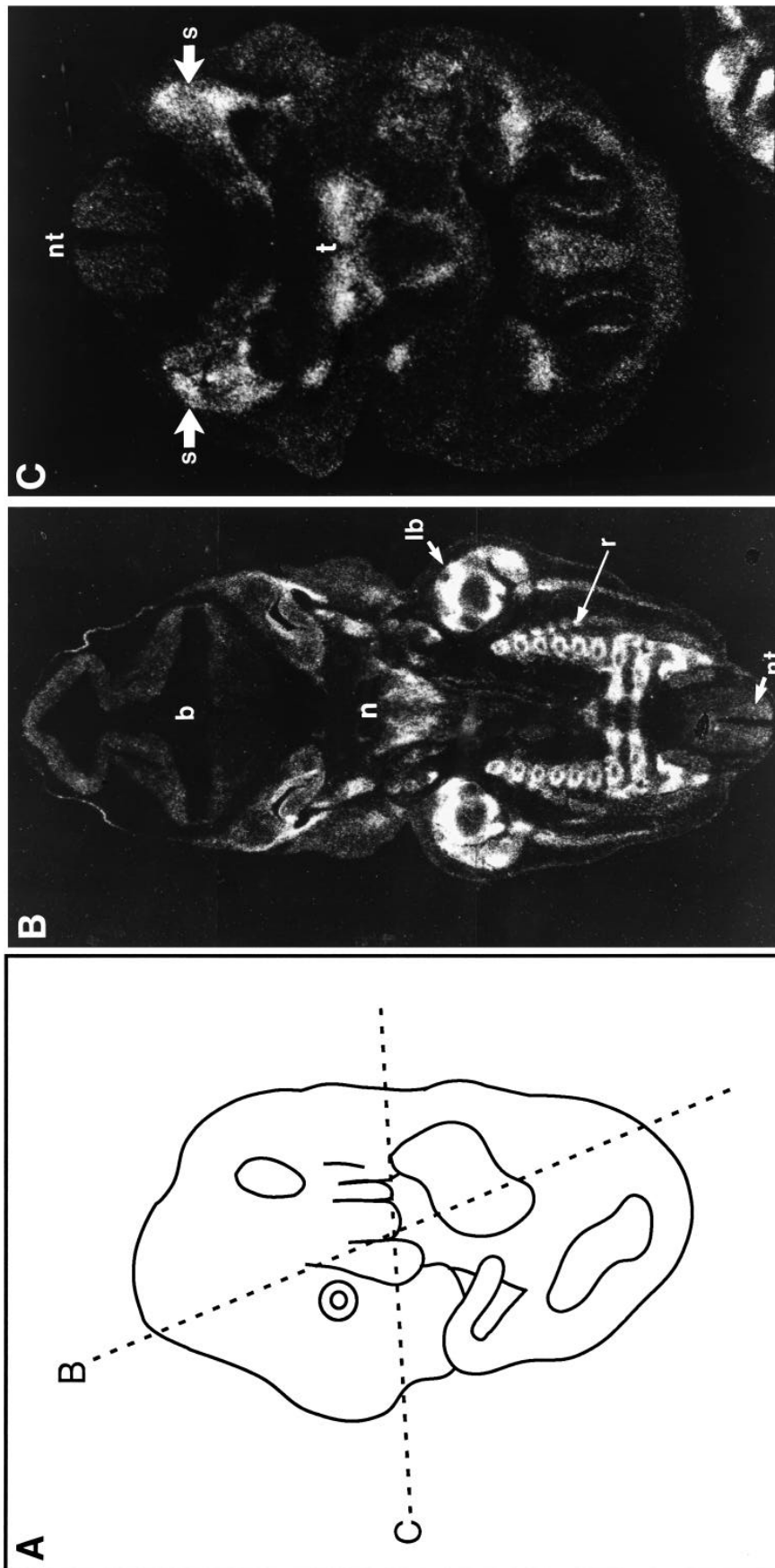


Fig. 5. Detection of scleraxis transcripts in day 11.5 p.c. mouse embryos by *in situ* hybridization. Transcripts for scleraxis were detected by *in situ* hybridization to frontal (B) and transverse (C) sections of a day 11.5 p.c. mouse embryo. The planes of section are shown in A. In C, the dorsal part of the embryo is on top. Scleraxis transcripts are present

in the condensing cartilage of the nose and face, in precursors of the limb bones and ribs, and in the sclerotomes. Note the absence of scleraxis expression in the central cores of the rib and long bone progenitors of the limbs. b, brain; lb, limb bud; n, nose; nt, neural tube; r, ribs; s, sclerotomes; t, tongue.

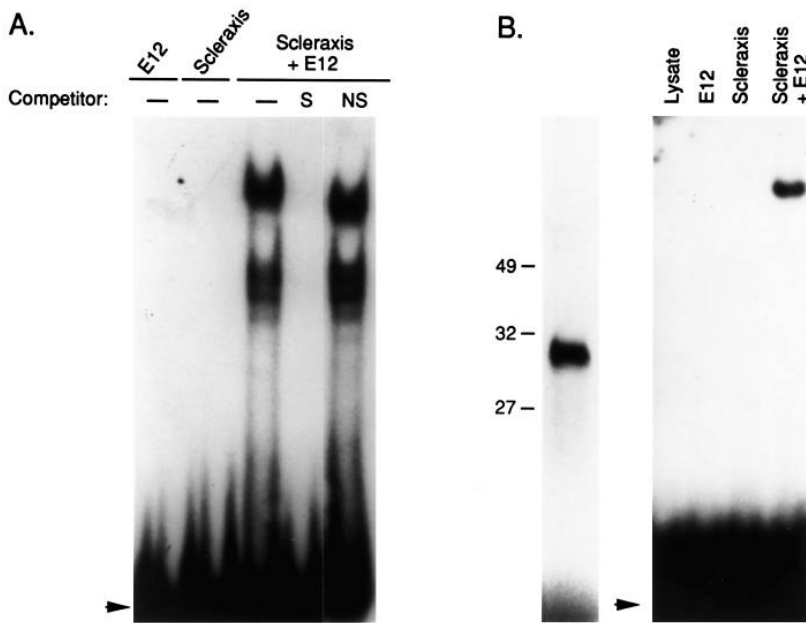


Fig. 6. DNA-binding activity of scleraxis protein. An oligonucleotide corresponding to the left E-box from the MCK enhancer was used as a probe in gel mobility shift assays with scleraxis and E12 expressed in bacteria (A) or translated in vitro (B). In A, assays were performed in the absence of competitor DNA (–) or in the presence of a 100-fold excess of the cognate oligonucleotide (S) or an oligonucleotide corresponding to the MEF2 site from the MCK enhancer (NS). Free probe is indicated by an arrowhead at the bottom in A and B. The lane on the left in B is an autoradiograph of [³⁵S]methionine-labeled scleraxis obtained from in vitro transcription/translation. M_r markers are to the left.

scleraxis plus GST-E12 was specific and was competed by the cognate DNA sequence but not by nonspecific DNA sequences. Among 10 E-box sequences tested, the MCK left E-box was bound most avidly by scleraxis plus E12, whereas others showed weak or no binding. Since neither scleraxis nor E12 showed appreciable binding to this sequence alone, we presume that the binding of the two together represents the formation of a scleraxis/E12 heterodimer.

When translated in a reticulocyte lysate, scleraxis showed an M_r ~30,000 (Fig. 6B). Like the bacterially expressed protein, the scleraxis in vitro translation product bound DNA only in the presence of E12 (Fig. 6B).

To test whether scleraxis had the potential to activate transcription through its DNA-binding site, we created a *CAT* reporter gene in which 4 tandem copies of the scleraxis-binding site were inserted upstream of the thymidine kinase basal promoter. This reporter was not expressed above background in transfected COS cells, which do not express scleraxis. However, in the presence of a scleraxis expression vector, the reporter gene was strongly transactivated (Fig. 7). The level of transactivation was comparable to that seen in the presence of an expression vector for MyoD (Davis et al., 1987), which is a potent transactivator. A *tk-CAT* reporter gene lacking the scleraxis-binding sites failed to be transactivated by scleraxis. We conclude that scleraxis can function as a strong transactivator.

Chromosomal mapping of the mouse scleraxis gene

The chromosomal location of *scleraxis* (locus designation *Scx*) was determined by interspecific backcross analysis using progeny derived from matings of [C57BL/6J×*Mus spretus*]F₁ × C57BL/6J mice. This interspecific backcross mapping panel has been typed for over 1600 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a cDNA probe. The 6.0 kb *M. spretus* *Eco*RI RFLP (see Materials and

Methods) was used to follow the segregation of the *Scx* locus in backcross mice. The mapping results indicated that *Scx* is located in the distal region of mouse chromosome 15 linked to *Tgn*, *Pdgfb* and *Wnt1*. Although 142 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 8), up to 171 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the

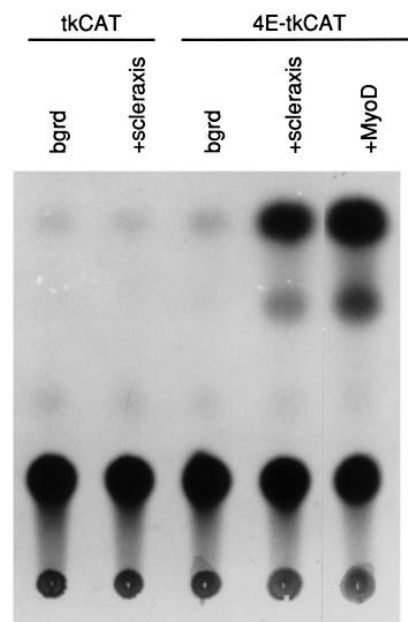


Fig. 7. Transcriptional activity of scleraxis. COS-1 cells were transiently transfected with 5 μ g of the indicated reporter gene and 5 μ g of scleraxis or MyoD expression vector, as described in Materials and Methods. An expression vector lacking a cDNA insert was transfected to determine background. 48 hours after transfection, cells were harvested and CAT activity was determined in aliquots of extract containing equivalent quantities of protein.

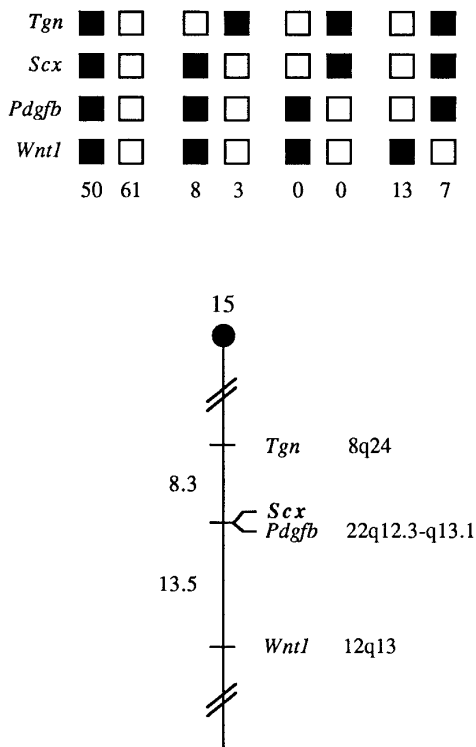


Fig. 8. *Scx* maps in the distal region of mouse chromosome 15. *Scx* was placed on mouse chromosome 15 by interspecific backcross analysis. The segregation patterns of *Scx* and flanking genes in 142 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 142 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*) F₁ parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of the *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 15 linkage map showing the location of *Scx* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centiMorgans are shown to the left of the chromosome, and the position of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order were centromere–*Tgn*–12/145–*Scx*–0/155–*Pdgfb*–23/171–*Wnt1*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± the standard error] were –*Tgn*–8.3±2.3–[*Scx*, *Pdgfb*]–13.5±2.6–*Wnt1*. No recombinants were detected between *Scx* and *Pdgfb* in 155 animals typed in common, suggesting that the two loci are within 1.9 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of chromosome 15 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (compiled by M. T. Davisson, T. H. Roderick, A. L. Hillyard and D. P. Doolittle, and provided from GBASE, a computerized database

maintained at the Jackson Laboratory, Bar Harbor, ME). *Scx* mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected to have an alteration in this locus (data not shown).

The distal region of mouse chromosome 15 shares a region of homology with human chromosomes 22q and 12q (summarized in Fig. 8). In particular, *Pdgfb* has been placed on human 22q12.3-q13.1. The tight linkage between *Pdgfb* and *Scx* in mouse suggests that *Scx* will also reside on 22q.

DISCUSSION

Because ubiquitous Class A bHLH proteins heterodimerize preferentially with cell-type-specific bHLH proteins (Murre et al., 1989b), we were able to use the yeast two-hybrid system, which detects protein-protein interactions, to screen for novel dimerization partners for E12 from a mouse embryo cDNA expression library. From this screen, we cloned a novel bHLH protein, scleraxis, which was expressed in mesenchymal precursors of cartilage and in connective tissue.

Skeleton formation occurs as a result of an ordered series of events in which mesenchymal cells aggregate into regions of high cell density that foreshadow the future skeletal elements. These cells then acquire a chondrogenic phenotype and secrete large amounts of extracellular matrix. Finally, these chondrogenic cells undergo ossification. Scleraxis is expressed in mesenchymal and chondrogenic cells that form the skeleton and it becomes downregulated when ossification is initiated.

Scleraxis transcripts were first detected within the sclerotomal region of the rostral-most somites between day 9.5 and 10.5 p.c. of embryogenesis. The somites form in a rostrocaudal progression by segmentation of the paraxial mesoderm, beginning at day 8 p.c. of mouse embryogenesis. Newly formed somites appear as an epithelial sphere, which becomes compartmentalized beginning at day 9.0 p.c. to form the sclerotome (which forms the axial skeleton), the myotome (the origin of skeletal muscle) and the dermatome (the origin of dermis). Scleraxis transcripts were not detected in the somites until after the sclerotome had formed and their expression appeared to be restricted to the sclerotomal compartment.

Cells from the sclerotome give rise to the ribs and vertebrae (Christ and Wilting, 1992). Scleraxis transcripts were expressed at high levels in the prevertebrae and in the rib primordia by day 10.5 p.c., which is well before the onset of chondrogenesis, which is normally initiated between 12.5 and 14.0 days p.c. (Rugh, 1990). Scleraxis was also expressed in chondrogenic precursors of the long bones of the limbs at a time when there are no recognizable cartilaginous structures in these regions. Within the bone primordia, scleraxis expression was confined to the periphery, which corresponds to the perichondrium and periosteum, which are a source of stem cells for appositional growth of bone and cartilage. Upon osteogenesis scleraxis expression declined to low levels. Scleraxis was not detected in cranial bones or their progenitors. These bones develop without a cartilaginous intermediate, suggesting that scleraxis is involved in chondrogenesis but not osteogenesis.

Scleraxis was also expressed in mesenchyme cells within the frontonasal region that give rise to facial structures and in mesenchymal cells in the mandibular region. These cells are derived from cranial neural crest cells (Noden 1988; Le

Douarin et al., 1993), which begin emigrating from the dorsal neural tube at the time of closure (about day 8.0 p.c.) and undergo an epithelial-to-mesenchymal transition at the time they begin to migrate (Lumsden et al., 1991; Sechrist et al., 1993). Scleraxis was not detected in neural crest cells prior to migration. Thus, it must be expressed after the mesenchymal neural crest cells reach their destinations in the frontonasal region and first branchial arch.

In addition to its expression in cartilage progenitors, scleraxis was expressed at high levels in tendons in the limbs, tongue and diaphragm, and in cartilage of bronchi. All of these cell types have in common the secretion of large amounts of extracellular matrix, such as types I and II collagens (Cheah et al., 1991). Whether scleraxis activates the expression of these types of extracellular matrix molecules remains to be determined.

Pax-1 is also expressed in the sclerotome (Deutsch et al., 1988; Koseki et al., 1993) and is required for the formation of the ventral parts of the vertebrae, the intervertebral discs and the proximal parts of the ribs (Wallin et al., 1994). The expression patterns of scleraxis and *Pax-1* in the sclerotome are overlapping, but clearly distinct. *Pax-1* is localized to the ventromedial region of the sclerotome adjacent to the notochord and is expressed earlier than scleraxis. When scleraxis first appears in the sclerotome, it is localized to the lateral region. Later, as the sclerotome begins to differentiate, scleraxis expression extends into the ventromedial region. Cells from the medial sclerotome migrate toward the notochord and give rise to the vertebral bodies and intervertebral discs, whereas cells from the lateral sclerotome form the pedicles and laminae of neural arches and ribs (Christ and Wilting, 1992). *Pax-1* expression is also restricted to chondrocytic derivatives of the somites, whereas scleraxis is expressed in chondrocytic derivatives throughout the embryo, as well as at a low basal level in many nonchondrocytic cell types.

The expression pattern of scleraxis in chondrocytic progenitors of bones is similar to that of the homeobox gene *CART1* (Zhao et al., 1993), which is expressed in condensed prechondrocytic mesenchymal cells and in early chondrocytes of cartilage primordia. Whether scleraxis collaborates with *Pax-1* or *CART1* to regulate chondrocytic gene expression remains to be determined.

The bone morphogenetic proteins (BMPs) have been shown to possess bone- and cartilage-inducing activity *in vivo* and *in vitro* and certain members of the BMP family are expressed at sites of bone formation in the embryo (reviewed in Reddi, 1994). However, little is known of the genetic pathways that direct cartilage and bone formation or of the mechanisms whereby the BMPs stimulate these processes. There is a particularly striking correlation between the expression patterns of BMP5 (King et al., 1994) and scleraxis during mouse embryogenesis. It will be of interest to determine whether these factors function in the same developmental pathway.

The activities of bHLH proteins are controlled by heterodimeric interactions and are influenced by partner availability (Kadesch, 1993). Dimerization of cell-type-specific bHLH proteins with E-proteins such as E12 results in the formation of heterodimers that are able to bind their specific target sequences and activate transcription (Murre et al., 1989b), whereas dimerization with the inhibitory HLH proteins of the Id family results in the formation of heterodimers that cannot bind DNA (Benezra et al., 1990). Members of the Id

family are not expressed in differentiating somites (Wang et al., 1992; Evans and O'Brien, 1993), suggesting that heterodimeric interactions between lineage-specific bHLH proteins and E-proteins can occur as the somites give rise to different cell lineages.

Like other cell-type-specific bHLH proteins, scleraxis binds the E-box consensus sequence as a heterodimer with E12. Scleraxis/E12 heterodimers do not bind to the majority of E-boxes that we tested, including the right E-box from the MCK enhancer, which binds members of the MyoD family with high affinity. Our results also demonstrate that scleraxis is a potent transactivator through its multimerized DNA-binding site. Although we do not yet know the target genes for scleraxis, based on its expression pattern, DNA-binding properties and transcriptional activity, scleraxis may regulate genes involved in establishing chondrogenic and dense connective tissue cell types.

It is particularly intriguing that compartmentalization of the somite is accompanied by the expression of myogenic bHLH proteins in the myotome and scleraxis in the sclerotome. In addition, we have recently identified a bHLH protein called paraxis, which is closely related to scleraxis and is expressed in newly formed epithelial somites and undifferentiated sclerotome (Burgess et al., *in press*). Another bHLH protein called Dermo-1 is expressed specifically in the dermamyotome and dermatome (L. Li and E. Olson, unpublished). Thus, the generation of myogenic, chondrogenic and dermal cell lineages from somitic mesodermal cells may involve independent pathways involving lineage-specific bHLH proteins. The expression of scleraxis in chondrogenic progenitors of the ribs is also interesting in light of the phenotype of *myf5*-null mice, which lack ribs (Braun et al., 1992). Whether this rib defect reflects cross-talk between myotomal and sclerotomal bHLH proteins or arises as a result of a disruption of the expression or function of scleraxis in the sclerotome is an interesting question for the future.

By comparison with other cell-type-specific bHLH genes such as the *MyoD* and *achaete scute* gene families, which regulate muscle and neural cell fates, respectively, it is tempting to speculate that *scleraxis* may play a role in the formation of chondrogenic cell types. In this regard, we have attempted to determine whether scleraxis is sufficient to activate the expression of chondrogenic or osteogenic markers in transfected fibroblasts, but have thus far not observed this type of activity (unpublished data). Gene inactivation through homologous recombination in transgenic mice should shed light on the possible functions of scleraxis in chondrogenic cell lineages.

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