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SOX9 Binds DNA, Activates Transcription, and Coexpresses with Type II Collagen during Chondrogenesis in the Mouse

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Two lines of evidence suggest that the *Sry*-related gene *Sox9* is important for chondrogenesis in mammalian embryos. *Sox9* mRNA is expressed in chondrogenic condensations in mice, and mutations in human *SOX9* are known to cause skeletal dysplasia. We show here that mouse SOX9 protein is able to bind to a SOX/SRY consensus motif in DNA and contains a modular transcriptional activation domain, consistent with a role for SOX9 as a transcription factor acting on genes involved in cartilage development. One such gene is *Col2a1,* which encodes type II collagen, the major structural component of cartilage. We have compared, in detail, the expression of *Sox9* and *Col2a1* during mouse development. In chondrogenic tissues the expression profiles of the two genes were remarkably similar. Coexpression was detected in some nonchondrogenic tissues such as the notochord, otic vesicle, and neural tube, but others such as heart and lung differed in their expression of the two genes. Immunohistochemistry using an antibody specific for SOX9 revealed that expression of SOX9 protein mirrored the distribution of *Sox9* mRNA. Our results suggest that SOX9 protein is involved in the regulation of *Col2a1* during chondrogenesis, but that this regulation is likely to depend on additional cofactors.

INTRODUCTION

During the development of the vertebrate skeleton, endochondral bone formation is initiated when mesenchymal cells condense and differentiate to form chondrocytes. These cells synthesize a framework of cartilage matrix in the approximate shape of future bones. The recent identification of gene defects responsible for skeletal abnormalities in mouse and man has shown that not only structural components of the cartilage extracellular matrix, but also growth and differentiation factors, signaling molecules, and transcription factors are important for proper morphogenesis during skeletal development (reviewed by Erlebacher *et al.,* 1995). However details of the mechanisms involved and the identities of many of the interacting molecules regulating chondrogenesis remain unknown.

Sox9 is a recently identified gene that may have a role in regulating chondrogenesis in mammals. This gene encodes one of a family of proteins that includes the mammalian Y-linked testis-determining factor, SRY (Gubbay *et al.,* 1990). Several SOX proteins have been shown to bind to DNA and activate transcription *in vitro* (van de Wetering *et al.,* 1993; Dubin and Ostrer, 1994; Hosking *et al.,* 1995; Kamachi *et al.,* 1995; Kanai *et al.,* 1996). This property and the tissue-specific expression pattern of some *Sox* genes (Koopman *et al.,* 1990; Stevanovic *et al.,* 1993; van de Wetering *et al.,* 1993; Uwanogho *et al.,* 1994; Connor *et al.,* 1995; Dunn *et al.,* 1995; Hosking *et al.,* 1995; Jay *et al.,* 1995; Kamachi *et al.,* 1995; Kanai *et al.,* 1996) indicate that they may regulate gene expression in specific cell lineages. A preliminary survey in which *Sox9* expression was examined in whole-mount mouse embryos has shown this gene to be expressed strongly in mesenchymal condensations before and during chondrogenesis, and in other tissues such as the central

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TABLE 1
Primers Used in the Construction of *Sox9* Deletion Mutants

Construct	Primers (5′–3′)	Primer position[a]
pGSox9p	Sox9A1 (GCGAATTCATGAATCTCCTGGACCCCTT)	302–321
	g10b (TTCCGTGGCCTCTTCGGCCTC)	863–882
pGR990	Sox9A3 (GGGAATTCGTGAAGAACGGACAAGCGG)	845–863
	Sox9B2 (CTTCTAGAAGGGTCTGGTGACCTGTGT)	1823–1805
pGR560	Sox9A2 (CCGAATTCGCGACCGCGGGCCACG)	1286–1301
	Sox9B2	
pGR300	Sox 9A2	
	Sox9B1 (AGTCTAGATGAAGGGGCTGTAGGAGAT)	1565–1547
pGR260	Sox9A4 (ACGAATTCCACTACAGGCCCTCCTAC)	1574–1591
	Sox9B2	

[a] Position shown relative to *Sox9* cDNA sequence described by Wright *et al.* (1995).

nervous system, otocysts, heart, and vibrissae (Wright *et al.*, 1995). The human *SOX9* gene is also expressed in differentiating chondrocytes, and mutation of the gene is associated with the skeletal malformation disorder campomelic dysplasia (CD; Foster *et al.*, 1994; Wagner *et al.*, 1994).

To delineate the potential role of *Sox9* in cartilage and bone development, it is important to determine its functional properties and identify molecules with which it may interact. The timing and sites of *Sox9* expression during mouse development (Wright *et al.*, 1995), and the skeletal dysmorphology associated with SOX9 mutations in CD patients (Lee *et al.*, 1972; Hovmoller *et al.*, 1977; Houston *et al.*, 1983; Foster *et al.*, 1994; Wagner *et al.*, 1994), indicate that a major function of SOX9 is to regulate a gene or genes important for chondrocyte differentiation and skeletal development. Candidates include genes expressed early in chondrocyte differentiation, such as those encoding cartilage extracellular matrix components (e.g., collagens, proteoglycans, cartilage matrix protein, and cartilage oligomeric matrix protein), growth factors (e.g., fibroblast growth factors, bone morphogenetic proteins, cartilage-derived growth factors, and parathyroid hormone-related peptide) and their receptors, signaling molecules (e.g., members of the hedgehog family), and other transcription factors.

The major extracellular matrix component of cartilage is type II collagen, which constitutes up to 60% of the total protein content. Type II collagen is a homotrimer of identical α1(II) chains, encoded in the mouse by the *Col2a1* gene. Transcription of *Col2a1* in mesenchymal cells precedes chondrocyte differentiation and is one of the hallmarks of chondrogenesis (von der Mark and von der Mark, 1977; Kravis and Upholt, 1985; Kosher *et al.*, 1986; Swalla *et al.*, 1988, Cheah *et al.*, 1991; Ng *et al.*, 1993). Type II collagen is important in maintaining the integrity of cartilaginous structures, and disruptions of its synthesis due to mutations of the *COL2A1* gene have been linked to human chondrodysplasias (reviewed by Vikkula *et al.*, 1994; Ritvaniemi *et al.*, 1995). Abnormal skeletal formation is also found in transgenic mice that express mutated type II collagen genes (Garofalo *et al.*, 1991; Vandenberg *et al.*, 1991; Metsäranta *et al.*, 1992; Li *et al.*, 1995).

In this study we show that SOX9 protein can bind to a SOX/SRY consensus sequence, copies of which are present in the regulatory regions of the human *COL2A1* gene. In addition, we identified a modular activation domain in the protein, suggesting a role for SOX9 as a transcriptional activator. Further, we have compared in detail the timing and sites of expression of the *Sox9* and *Col2a1* genes during mouse embryogenesis. We show, by *in situ* hybridization, a concordance of expression of these two genes at sites of chondrogenesis, consistent with a role for *Sox9* as a direct or indirect regulator of *Col2a1*.

MATERIALS AND METHODS

Cloning of Sox9 Deletion Mutants

A construct (pGSox9p) containing the *Sox9* open reading frame and the 3′ untranslated region was cloned as a fusion of a 583-bp PCR fragment generated using the primers Sox9A1 and g10b (Table 1) and a 1.6-kb *Bss*HII/*Xho*I fragment of *Sox9* cDNA (Wright *et al.*, 1995) into the expression vector pGAL0 (Kato *et al.*, 1990). *Sox9* deletion mutants were generated by cloning PCR fragments generated from the *Sox9* cDNA in frame into the same vector. PCR primers used for this purpose are described in Table 1. Inserts of constructs other than pGR990 were sequenced completely, and all insert/vector junctions were sequenced using a GAL4-specific primer (5′-CGACATCATCATCGGAAGAG-3′) to verify the reading frame.

Electrophoretic Mobility Shift Assay (EMSA)

To generate a source of SOX9 protein suitable for EMSA analysis, 20 μg of a plasmid containing the coding sequence for the first 147 amino acids of the yeast transcriptional activator GAL4 (GAL0; Kato *et al.*, 1990) fused to the *Sox9* coding sequence (Wright *et al.*, 1995) was transfected into COS-1 cells using Lipofectamine (Gibco BRL) as described (Kent *et al.*, 1996). Nuclear extracts were prepared from GAL0-transfected and GALSOX9-transfected cells using a

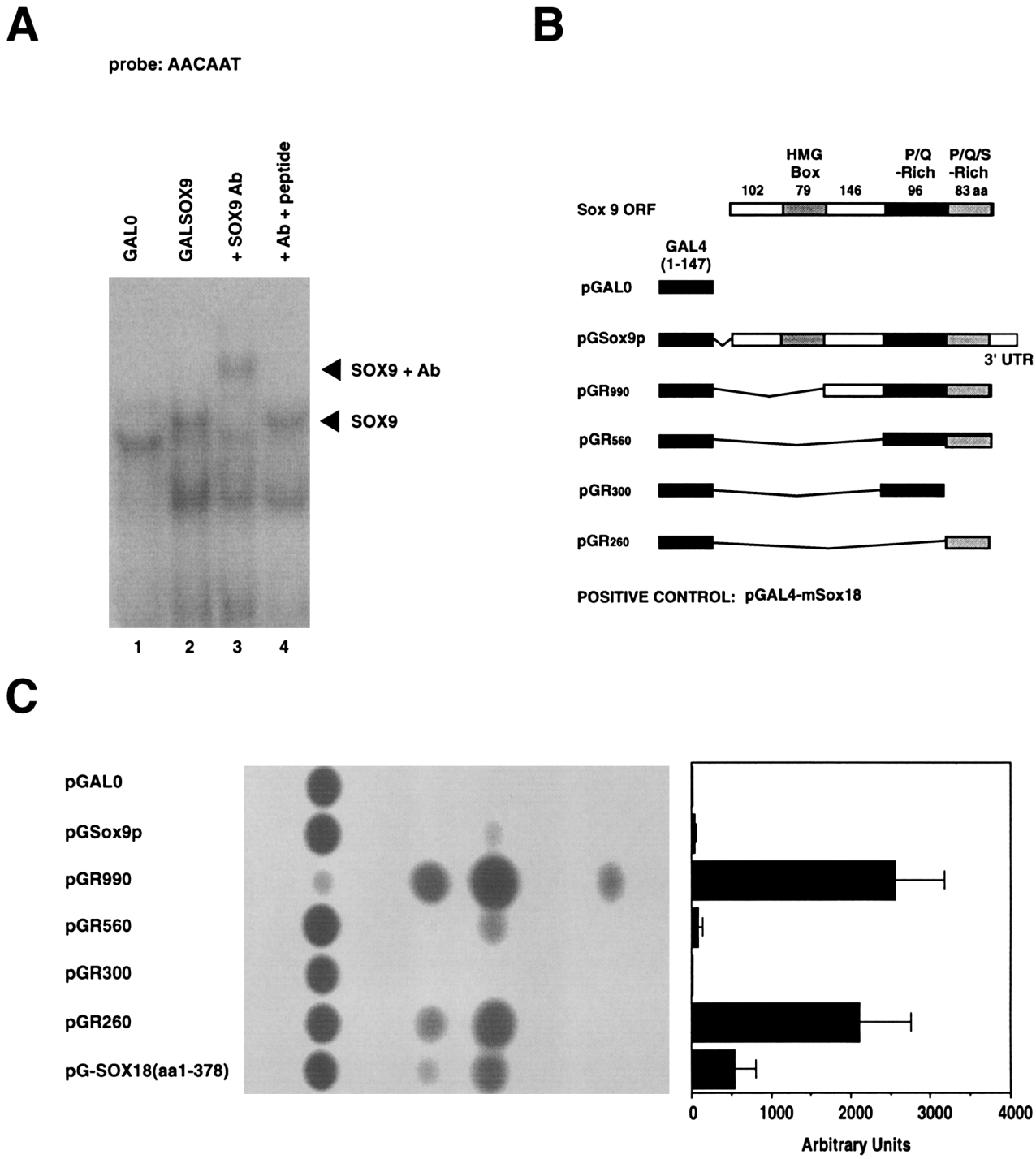


FIG. 1. DNA binding and transcriptional activation by SOX9 protein. (A) SOX9 binds to the SOX/SRY consensus AACAAT. EMSA analysis: lane 1, an extract from GAL0-transfected COS-1 cells incubated with the probe does not produce a specific retarded band; lane 2, an extract from GALSOX9-transfected cells produces a retarded band specific for SOX9; lane 3, the identity of the SOX9 band is confirmed using anti-SOX9 polyclonal antiserum, which produces a ''supershift''; lane 4, this supershift is abolished by incubating the probe, extract, and antibody with the immunogenic peptide which competes for binding to the antibody and prevents it binding to SOX9 and producing the supershift. The positions of the SOX9 band and the ''supershifted'' band are indicated. (B, C) Activation of transcription by SOX9. (B) Constructs for GAL4 transcriptional activation assays containing the full-length *Sox9* open reading frame or fragments thereof fused to the GAL4 DNA-binding domain. (C) The extent of transcriptional activation by each construct is indicated by the CAT assay results and the mean of triplicate assays is shown with bars indicating standard error.

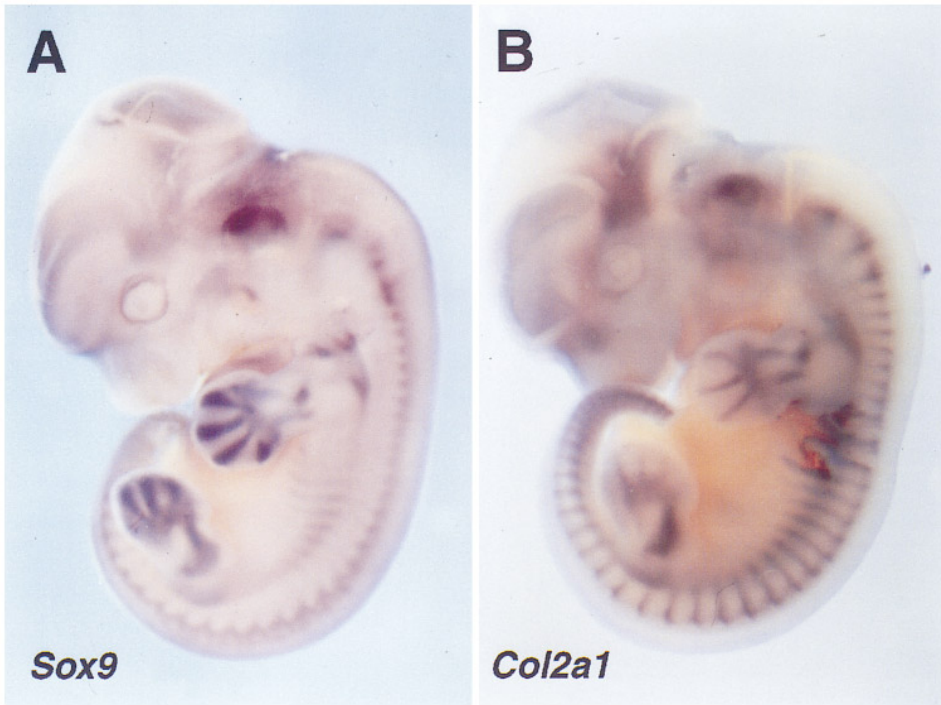


FIG. 2. Whole-mount *in situ* hybridization of 12.5 dpc fetuses showing the similarity of expression of *Sox9* (A) and *Col2a1* (B) in skeletal structures.

method based on that described by Osborn *et al.* (1989). Briefly, after washing the cells in buffer A, 10^7 cells were resuspended in 500 μl buffer A and 1 ml buffer A/0.1% NP-40 was added. After mixing gently, nuclei were collected by centrifugation at 2500 rpm for 3 min at 4°C in a microfuge and then resuspended in 100 μl buffer C. After vortexing, incubation on ice for 10 min, and centrifugation at 13,000 rpm for 10 min at 4°C in a microfuge, the supernatant containing nuclear proteins was stored at −70°C. Binding reactions (Schreiber *et al.*, 1989) were performed in the presence of 2 μg salmon sperm DNA and 1 μg/μl BSA. An extract from GAL0-transfected cells was used as a background DNA-binding activity control. Briefly, 4 μg of nuclear extract was incubated initially with all components except probe for 30 min on ice. After the addition of 1 ng gel-purified, double-stranded probe (sense strand: 5′-GATCCGGACTAATAAACAATAAAGTCGACG-3′), the reactions were incubated on ice for a further 30 min and loaded onto a nondenaturing 5% polyacrylamide, 0.8× TBE gel. Sixteen-centimeter gels were run at 10 V/cm and the dried gels were exposed to Fuji RX X-ray film. Variations to this standard procedure included adding 0.5 μg affinity-purified anti-SOX9 polyclonal antiserum (Kent *et al.*, 1996) or adding antibody plus 1 μg of the immunogenic SOX9 peptide.

Transfection of Mammalian Cells and Chloramphenicol Acetyl Transferase (CAT) Assays

COS-1 cells at 60–70% confluence were transiently transfected with 1.5 μg of chimeric *GAL4-Sox9* plasmids and 3.5 μg of reporter plasmid (pG5E1bCAT, containing five GAL4 binding sites upstream of the CAT gene) per well of a six-well plate, using DOTAP (Boehringer-Mannheim). Between 40 and 48 hr after transfection, cells were harvested and the ability of SOX9 to activate transcription was assayed by measuring CAT activity (Gorman *et al.*, 1982). Constructs were assayed in three independent experiments.

In Situ Hybridization

Antisense and sense probes for *in situ* hybridization were generated from clones pNJ61, pEL111, and pSox9.5a. pNJ61 is a 500-bp fragment covering exon 1 to exon 5B of *Col2a1* obtained by RT-PCR using oligonucleotides 5′-TCTCCTGCCTCCTCCTGCTC-3′ and 5′-CTCCATCTCTGCCACGGGGT-3′ subcloned in pBluescribe (Stratagene). pEL111 is a 0.6-kb genomic fragment of *Col2a1* containing exon 1 (Cheah *et al.*, 1991). pSox9.5a (Wright *et al.*, 1995) contained 500-bp of sequence 3′ to the HMG box of *Sox9* not including the poly(A) sequence, subcloned in pBSKSII$^-$ (Stratagene).

Whole-mount *in situ* hybridization was carried out using 12.5 days post coitum (dpc) Swiss outbred mouse embryos as described by Wright *et al.* (1995), using pSox9.5a and pEL111 probes. For *in situ* hybridization to sections, CBA/n mouse embryos at 7.5 to 18.5 days were fixed in 4% paraformaldehyde and processed as described (Cheah *et al.*, 1991). pNJ61 and pSox9.5a RNA probes labeled with [α-^{35}S]UTP (1000 Ci/mmol, Amersham) were generated by *in vitro* transcription and used to localize transcripts in embryonic tissues. The number of UTP molecules per strand of antisense RNA probe generated from pNJ61 and pSox9.5a are 110 and 119, respectively; therefore, the intensities of hybridization signals of the two riboprobes are comparable. pNJ61 and pSox9.5a were hybridized to consecutive adjacent sections covering 24–48 μm. Specific hybridization was achieved by treating the sections with 20 μg/ml RNase

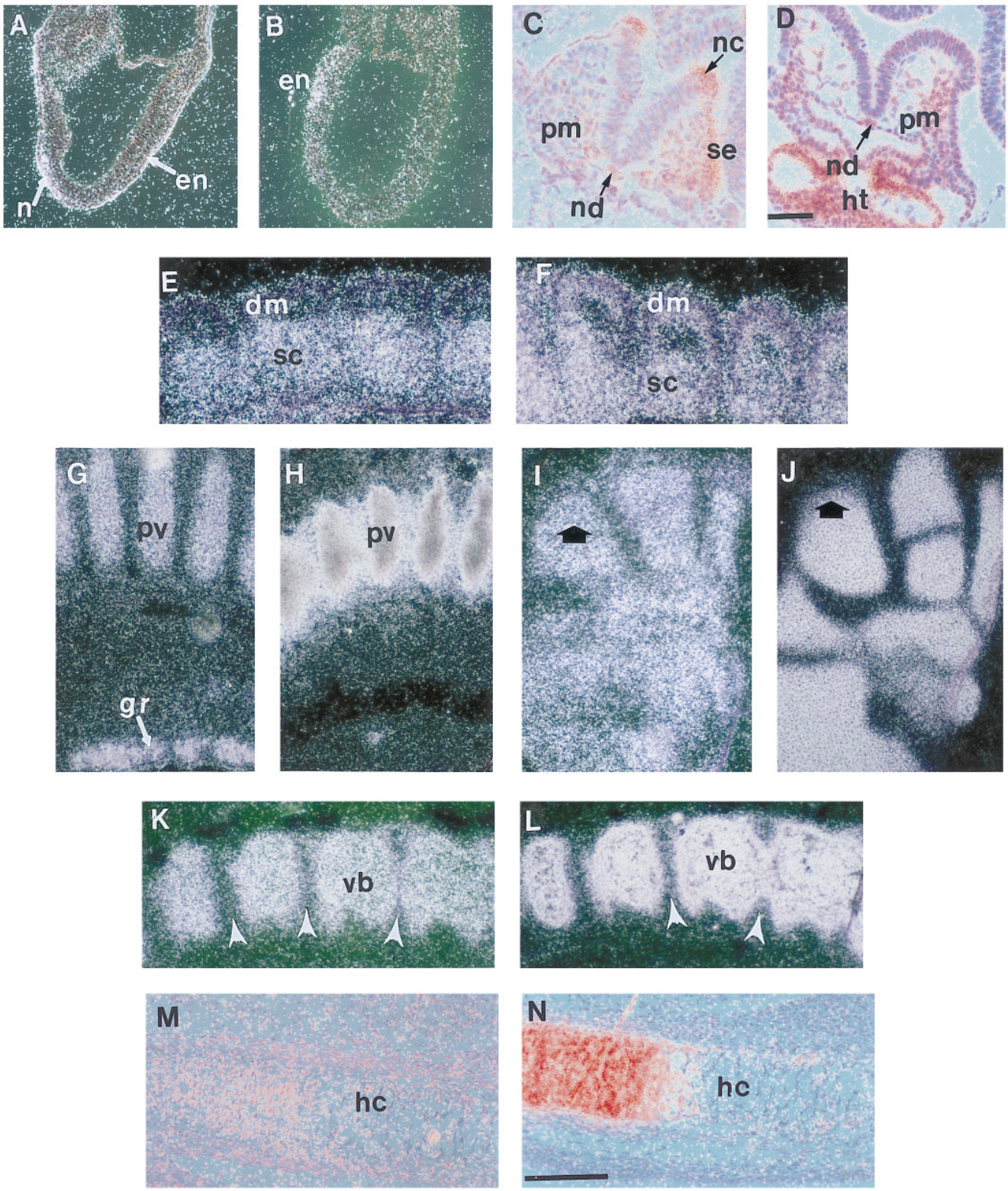


FIG. 3. Coexpression of *Sox9* and *Col2a1* in the early embryo and chondrogenic tissues. *Sox9* expression is shown in A, C, E, G, I, K, and M (left-hand side of each pair of photomicrographs) and *Col2a1* in B, D, F, H, J, L, and N (right-hand side of each pair). Each pair of illustrations was taken from sections approximately 24–48 μm apart within the same specimen. (A) *Sox9* expression in the notochordal plate (n) at 7.5 dpc. (B) 7.5 dpc embryo hybridized with *Col2a1* probe showing expression limited to a small patch of primitive endoderm

A and 50 u/ml RNase T_1 at 37°C for 45 min and washing at 65°C in 2× SSC, 50% formamide, 20 m*M* dithiothreitol. Autoradiography and photography of sections were as described previously (Cheah *et al.,* 1991).

Immunohistochemistry

Immunohistochemistry was performed on frozen sections of mouse embryos. Embryos were dissected into PBS, fixed in 4% paraformaldehyde for 4 hr or overnight at 4°C, and saturated with 15% glucose in PBS overnight at 4°C. After incubation in graded mixtures of 15% glucose/PBS and OCT compound (Tissuetek), embryos were embedded in OCT in cryomoulds (Tissuetek) and frozen on dry ice for 30 min. Ten-micrometer sections were cut on a cryostat at −20°C. Sections were applied to SuperFrost/Plus slides (Menzel Glaser, Germany) and allowed to dry thoroughly before staining.

All steps in the staining procedure were performed in a humidified chamber at 4°C. Sections were first rinsed 2× with PBS then bleached with 0.6% H_2O_2 in PBS for 20 min. Sections were then blocked with 10% fetal calf serum/0.1% Triton X-100 in PBS for 30 min. The specificity of the anti-SOX9 antibody has been established previously (Kent *et al.,* 1996). Affinity-purified anti-SOX9 antiserum was diluted to 10 μg/ml in PBS/Triton X-100 and applied to the sections overnight. The sections were then washed in PBS/Triton X-100 and a biotinylated anti-rabbit Ig (Amersham) was diluted to 10 μg/ml and applied to the sections overnight. After washing in PBS/Triton X-100 then PBS alone, a horseradish peroxidase (HRP)-conjugated avidin/biotin complex (ABC) reagent (Vector Laboratories) was applied to the sections for 30 min. After washing in PBS, HRP was developed using 2.5 μg/ml 3,3′-diaminobenzidine, 0.08% $NiCl_2$, and 0.03% H_2O_2. Sections were dehydrated, cleared, and mounted in DPX mounting medium. Photomicrographs were taken on an Olympus AX70 microscope using Kodak 64T Ektachrome film.

RESULTS

SOX9 Can Bind DNA and Is a Transcriptional Trans-Activator

The sequence AACAAT has been determined as the optimal binding site for SRY (Harley *et al.,* 1994), SOX5 (Denny *et al.,* 1992), and SOX17 (Kanai *et al.,* 1996), and it has been shown that this sequence can bind SOX2 (Kamachi *et al.,* 1995) and SOX6 (Connor *et al.,* 1995). Copies of this sequence are present in the regulatory regions of the human *COL2A1* gene (Metsäranta *et al.,* 1991), suggesting that it might mediate binding of SOX9 to this gene and activate transcription. EMSA analysis was carried out to investigate whether SOX9 can bind to this consensus. Specific binding activity with this probe was observed with extracts of GAL-SOX9-transfected COS-1 cells, but not with extracts of GAL0-transfected cells (Fig. 1A). The identity of the SOX9-specific band was confirmed by including affinity-purified anti-SOX9 polyclonal antiserum (Kent *et al.,* 1996) in some binding reactions. This produced a ''supershift'' of the SOX9 band, which was abolished by incubating the binding reaction plus antibody with the immunogenic peptide used to raise the antibody (Fig. 1A). These results demonstrate that SOX9 binds to the consensus sequence AACAAT.

Eukaryotic transcription factors usually consist of modular DNA binding and transcriptional activation or repression domains (Ptashne, 1988; Mitchell and Tjian, 1989). Consistent with a role as a transcriptional activator, the SOX9 protein contains two domains which are rich in proline, glutamine, or serine residues and which are distinct from the HMG box DNA binding domain (Wright *et al.,* 1995). To determine whether SOX9 is able to activate transcription and to define the domains of activation, distinct SOX9 protein domains were assayed using the GAL4 hybrid system in transfected mammalian cells (Lillie and Green, 1989).

A full-length SOX9 construct (pGSox9p; Fig. 1B) was able to activate transcription, albeit weakly (Fig. 1C), raising the possibility that the presence in this construct of a second DNA binding domain, the HMG box, affected the assay. To examine this possibility, the HMG box was excluded by assaying only the 3′ coding sequence (pGR990; Fig. 1B). This construct was able to stimulate the transcription of the reporter gene to a very high level (Fig. 1C), indicating that SOX9 contains at least one activation domain downstream of the HMG box.

Further experiments were performed to delimit the activation domain(s). A region exceptionally rich in proline and glutamine residues (amino acids 329–421) was considered most likely to be responsible for the transcriptional activation, by analogy with the composition of activation domains in the B-cell-specific factor OTF-2 (Clerc *et al.,* 1988), the human Sp1 transcription factor (Courey and Tjian, 1988), and members of the CTF/NF-I family (Santoro *et al.,*

cells in the anterior region (en). (C, D) Expression of both genes is detected in the cranial paraxial mesenchyme (pm) and notochord (nd) of 8.5 dpc embryos (see also coexpression in 9.5 dpc notochord, Figs. 6A and 6B). The *in situ* hybridization signal for *Sox9* transcripts is strong in the crest of the neural plate (nc), the surface ectoderm (se, presumptive placodal epithelium), and the subjacent neural crest cells (C). Cardiogenic mesoderm (ht) shows strong *Col2a1* expression (D). (E, F) Coexpression in the sclerotome (sc) of the lower trunk somites of a 9.5 dpc embryo. Weaker *Sox9* expression is found in the dermamyotome (dm). (G, H) Mesenchymal condensation for prevertebrae (pv) expresses both *Sox9* and *Col2a1* mRNAs. Strong *Sox9* expression is found in the urogenital ridge (gr) of this 11.5 dpc embryo (sex unknown). (I, J) Expression of *Sox9* and *Col2a1* in the chondrogenic mass of the tarsals and metatarsals in the hind limb of a 15.5 dpc fetus. The arrows point distally. (K, L) Coexpression of both genes in the chondrocytes of the lower trunk vertebrae (vb) of a 14.5 dpc embryo and diminished level of expression in the intervertebral tissues (arrowheads). (M, N) Relatively stronger expression of *Col2a1* compared to *Sox9* in the proliferating chondrocytes of the basioccipital bone at 16.5 dpc. In hypertrophic chondrocytes (hc) *Col2a1* mRNAs were diminished and *Sox9* transcripts were almost at background levels. Bar = 50 μm (A–D) and 100 μm (E–N).

1988). Present within this region is a stretch of 39 amino acids composed exclusively of proline (15), glutamine (17), and arginine residues (7). Surprisingly, this region was able to transactivate only weakly (pGR300; Figs. 1B and 1C). Instead, the C-terminal fragment (pGR260, amino acids 424–507; Fig. 1B) was able to stimulate transcription to a very high level, comparable to the strong transcriptional activators SOX18 (Hosking *et al.*, 1995; Fig. 1C) and Sp1 (Courey and Tjian, 1988; data not shown). These results demonstrate the presence of a potent modular activation domain within the terminal 83 amino acids of the SOX9 protein.

Sox9 and Col2a1 Are Coexpressed in Skeletogenic Tissues

A comparison of the distribution of the *Sox9* and *Col2a1* transcripts by whole-mount *in situ* hybridization of midgestation mouse fetuses revealed a widespread concordance of gene expression in cartilaginous tissues (Fig. 2). Expression of these two genes was examined in detail by *in situ* hybridization on sections of mouse embryos of various stages. The presence of SOX9 protein was confirmed at many *Sox9* mRNA expression sites by immunohistochemistry on cryosections of mouse embryos. Coexpression of *Sox9* and *Col2a1* was first noted in the cranial mesenchyme of embryos at the early somite stage (8.5 dpc). *Sox9* was expressed strongly in the mesenchyme subjacent to the surface ectoderm and the epidermal placodes at the mid- and hindbrain level, which presumably consists of migrating neural crest cells that contribute to the facial skeleton (Fig. 3C). Moderate expression was also found in the paraxial mesoderm (Fig. 3C). *Col2a1* expression in the cranial mesenchyme was mostly confined to the paraxial mesoderm with lower levels of expression in the neural crest cell population (Fig. 3D). Both *Sox9* and *Col2a1* were expressed at later stages in the frontonasal mesenchyme, cranial paraxial mesenchyme, branchial arch mesenchyme, and the dispersing sclerotome of the somites (Figs. 3E and 3F). SOX9 protein was also observed in the dispersing sclerotome of the somites at 10.5 dpc (Fig. 4A).

Sox9 and *Col2a1* were strongly expressed when the prechondrogenic mesenchyme condensed into cartilage primordia throughout the developing skeleton (Fig. 2). For example, the prevertebrae derived from the condensation of sclerotomal cells clearly expressed both genes (Figs. 3G and 3H) and SOX9 protein expression was also confirmed in these structures (Figs. 4B and 4C). Transcription of both genes and expression of SOX9 protein (Fig. 4D) were maintained as the prechondrocytes differentiated into immature and proliferating chondrocytes. *In situ* hybridization revealed the presence of *Sox9* and *Col2a1* mRNAs in the perichondrium and the pre-hypertrophic chondrocytes of fetal cartilages such as the hind limb cartilages (Figs. 3I and 3J), vertebral bodies (Figs. 3K and 3L), cartilage in the skull, larynx, sternum, limb girdles, and Meckel's cartilage. Both transcripts and SOX9 protein were absent or at very diminished levels in hypertrophic chondrocytes in the centrum

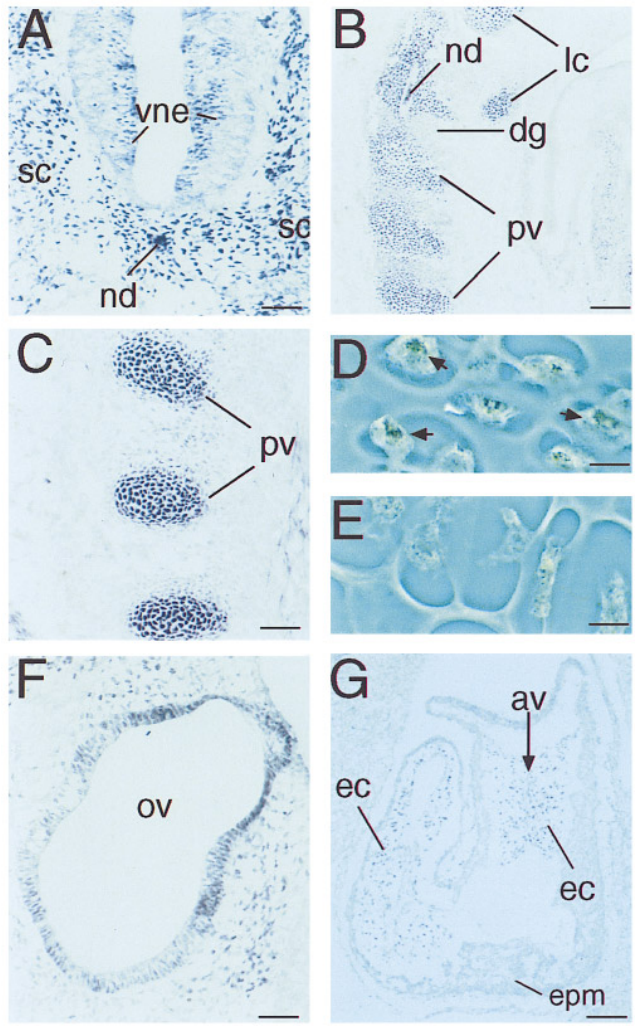


FIG. 4. Immunohistochemical analysis of SOX9 protein expression. (A) A transverse section of a 10.5 dpc mouse embryo. SOX9 protein is present in the notochord (nd), the sclerotome (sc) of the somites and the ventricular neuroepithelial layer of the spinal cord (vne). (B) A parasagittal section of a 13.5 dpc mouse embryo showing SOX9 protein in the prevertebrae (pv). The SOX9-expressing notochord (nd) is also seen, while the dorsal root ganglia (dg) between the prevertebrae are negative. SOX9 is also expressed in the mesenchymal condensations of the laryngeal cartilages (lc). (C) A parasagittal section through a 13.5 dpc embryo, more lateral to that in B, showing SOX9 expression in the vertebral bodies undergoing chondrocytic differentiation, but not yet hypertrophy. (D) High-power view showing the strong expression of SOX9 in proliferating chondrocytes from a hind limb bone of a 17.5 dpc embryo. Arrows show SOX9 protein in the nuclei of these cells. (E) High-power view of hypertrophic chondrocytes from the same bone as in D, showing that the expression of SOX9 protein is downregulated upon hypertrophy of the chondrocytes. (F) A parasagittal section through a 10.5 dpc embryo showing that SOX9 is expressed in the epithelium of the otic vesicle (ov). (G) A parasagittal section of a 10.5 dpc embryo showing SOX9 protein in the endocardial cushions of the developing heart (ec) flanking the atrioventricular canal (av). SOX9 protein is absent from the epimyocardium (epm). Bar = 100 μm (A, B), 50 μm (C, F, G), and 25 μm (D, E).

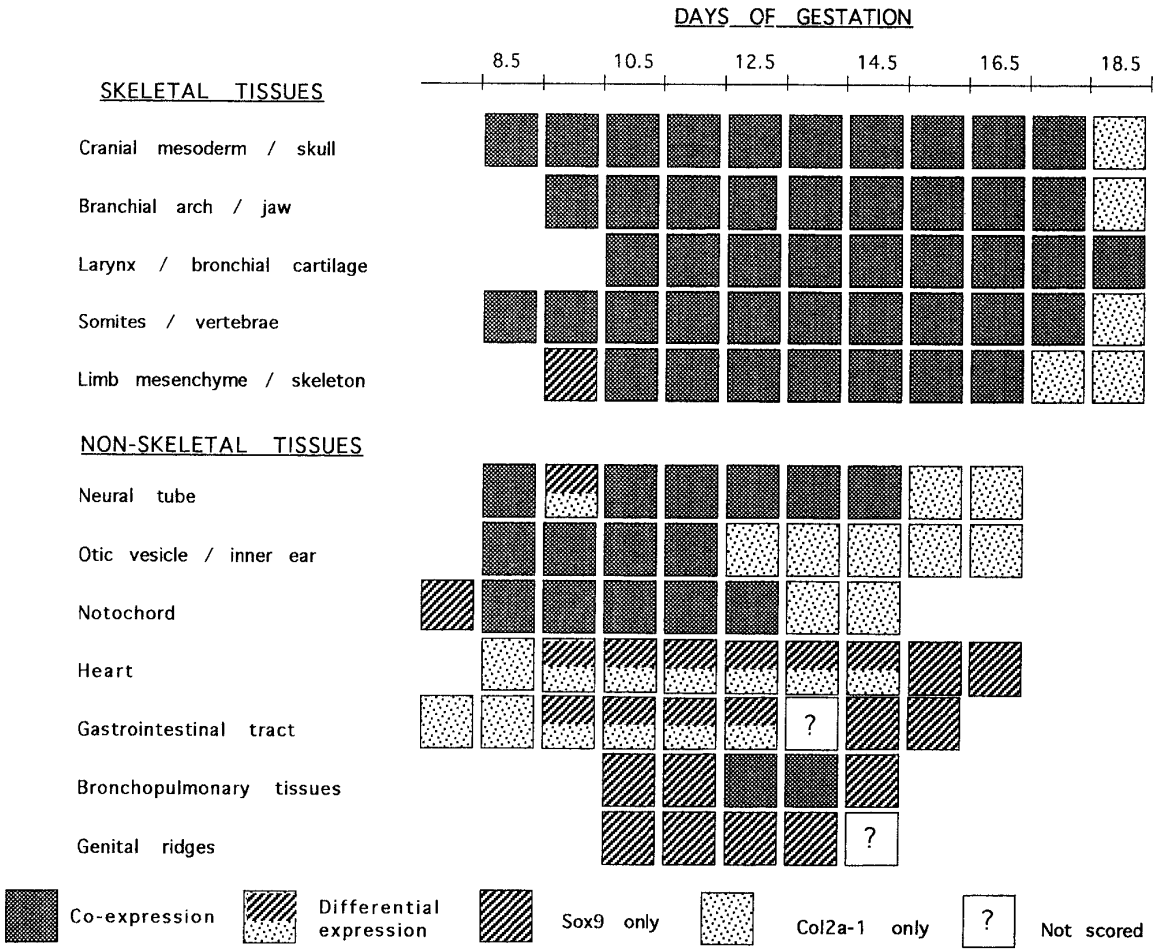


FIG. 5. Summary of the expression profiles of *Sox9* and *Col2a1* in skeletal and nonskeletal tissues during mouse development.

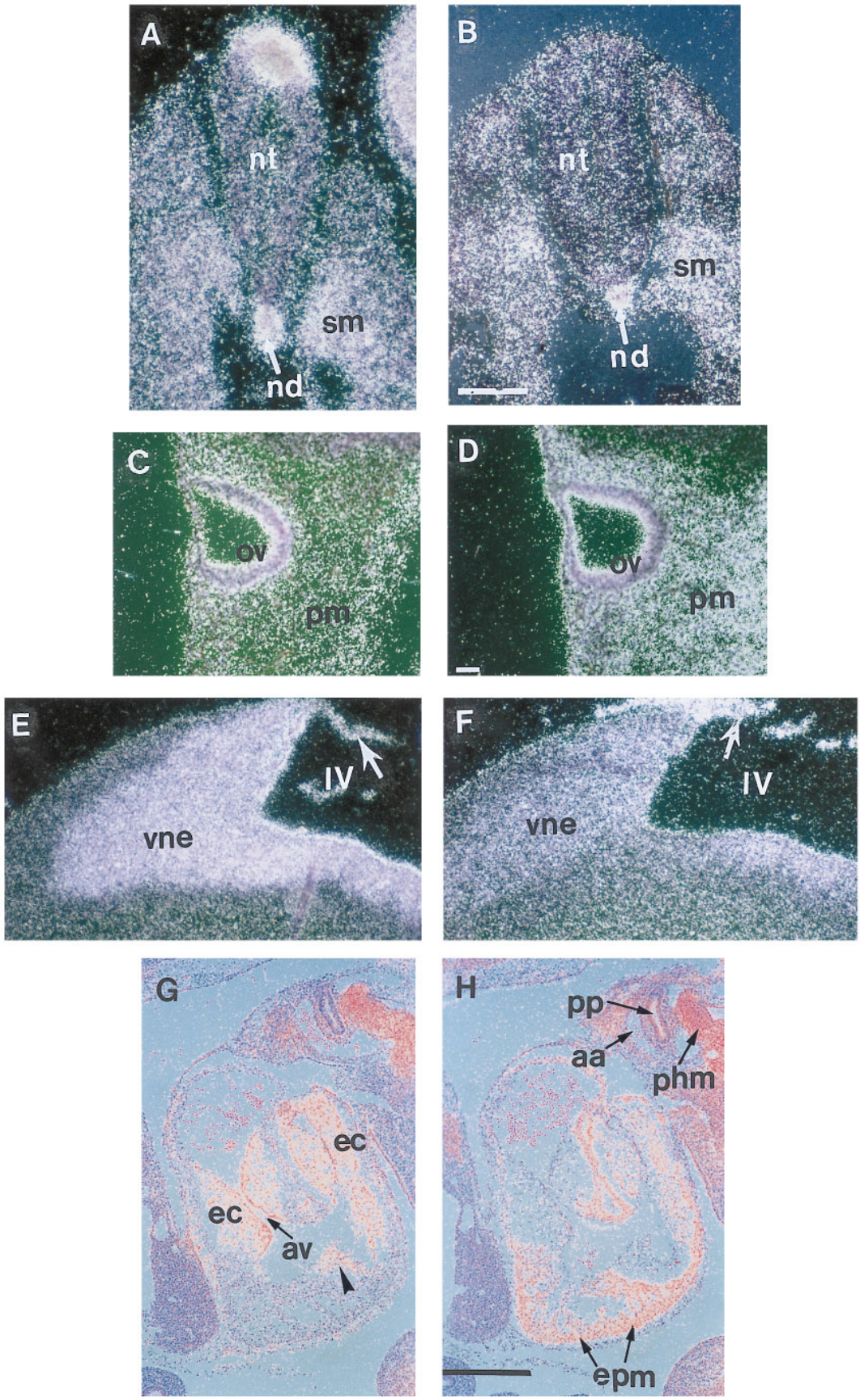
of the vertebrae and in the preossified regions of the cartilage in the chondrocranium, ribs, otic capsule, and limb bones (Figs. 3M and 3N, 4E, and data not shown). From 13.5 dpc onward, expression of *Sox9* and *Col2a1* was also found in some osteogenic tissues of membranous bones such as the cranium and the facial and jaw skeleton. *Sox9* mRNAs were concentrated mostly in the endocranium in contrast with the more uniformly distributed *Col2a1* transcripts. During late fetal development (17.5–18.5 dpc), *Sox9* expression progressively diminished in most skeletal tissues and by 18.5 days, *Sox9* expression was found only in small clusters of chondrocytes in the femur, vertebral bodies, larynx, and some osteoblasts in the mandible and maxilla. *Col2a1* expression continued during this period.

In summary, coexpression of *Sox9* and *Col2a1* mRNA and of SOX9 protein was observed during the differentiation and maturation of chondrocytes and osteogenic tissues (Fig. 5). However, during late fetal development, *Sox9* mRNA and protein expression in mature cartilage and membranous bones is downregulated in advance of *Col2a1*.

Sox9 and Col2a1 Are Coexpressed in a Subset of Nonskeletal Tissues

The concordant expression of *Sox9* and *Col2a1* mRNA and SOX9 protein was not found exclusively in chondrogenic and osteogenic tissues. Coexpression was also observed in the notochord and otic vesicles and at specific stages of central nervous system (CNS) development. In general, as in skeletogenic tissues, *Col2a1* was activated at the same stage as or shortly after *Sox9*, and *Col2a1* expression tended to persist beyond the extinction of *Sox9*.

Notochord. *Sox9* mRNA was first detected in the node, the early notochordal plate, and the mesoderm immediately lateral to the node of the late primitive streak-stage embryo, before the onset of expression of *Col2a1* (Figs. 3A and 3B). Expression of *Col2a1* lagged behind that of *Sox9* and was first detected in the nascent notochord of the early-somite-stage embryo (8.5 dpc; Fig. 3D). *Sox9* and *Col2a1* were coexpressed in the notochord until 12.5 dpc (Figs. 3C and 3D, 4A and 4B, 5, and 6A and 6B), after which time *Sox9* transcripts were not detected. However, SOX9 protein expres-



sion was confirmed in the notochord at 10.5 and 13.5 dpc (Figs. 4A and 4B), demonstrating that SOX9 protein expression persisted beyond that of *Sox9* mRNA.

Otic vesicles. Expression of *Sox9* was first detected in the epidermal placodes that precede the invagination of the otic vesicle (Fig. 3C). Once invagination has occurred, both *Sox9* and *Col2a1* mRNA and SOX9 protein were expressed in the epithelium of the vesicles (Figs. 4F and 6C and 6D). In contrast to *Col2a1*, *Sox9* transcripts were not detected in the sensory epithelium of the inner ear at 11.5–17.5 dpc (data not shown).

Central nervous system. *Sox9* and *Col2a1* were expressed in different dorsoventral regions of the early neural tube, but came to be coexpressed in later stages of CNS development. *Sox9* was first expressed in the crest region of the cephalic neural plate at 8.5 dpc (Fig. 3C). In the closed neural tube, *Sox9* expression was strongest in the roof plate and the alar plate (Fig. 6A), and *Col2a1* expression was generally low (Fig. 6B). Coexpression of these genes was found in the neuroepithelium of the forebrain at 8.5–9.5 dpc. At later stages (10.5–16.5 dpc), *Sox9* and *Col2a1* were coexpressed in the ventricular layer of the forebrain, midbrain, and hindbrain (Figs. 6E and 6F) and in the roof plate of the lower hindbrain and cervical spinal cord. SOX9 protein was also detected in the ventricular neuroepithelial layer of the spinal cord (Fig. 4A). Expression of *Sox9* was extinguished by 15.5–16.5 dpc but *Col2a1* transcripts persisted in the hindbrain neuroepithelium.

Differential Expression of Sox9 and Col2a1

Despite extensive coexpression of *Sox9* and *Col2a1* during development, particularly in skeletogenic tissues, many examples of differential expression of the two genes were found in other fetal tissues at various stages. These are summarized in Table 2 and Fig. 5. In some mesoendodermal organs, *Sox9* and *Col2a1* were expressed in tissue types derived from different germ layers. For example, *Sox9* was not expressed in the endoderm of the pharynx where *Col2a1* was expressed. In the respiratory tract, *Sox9* was expressed in the bronchial epithelium and the mesenchyme of the lung bud but *Col2a1* transcripts were found only in the bronchial epithelium and not in the mesenchyme at 12.5 dpc. A striking example of differential expression of the two genes was seen in the heart. At early somite stage (8.5 dpc), *Sox9* transcripts were absent when *Col2a1* was expressed strongly in the cardiogenic mesoderm (Figs. 3C and 3D). *Sox9* expression was found during the differentiation of the heart tissues and was restricted initially to the thin layer of endocardium in the atrium at 9.5 dpc. Subsequently, at 11.5 and 12.5 dpc *Sox9* was found in the endocardium of the heart chambers, in the endocardial cushions of the heart valves, and in the atrioventricular canal (Fig. 6G). Differential expression of these genes was confirmed by the observation that SOX9 protein is expressed only in the endocardial cushions of the heart at 10.5 dpc (Fig. 4G). In contrast, *Col2a1* was expressed in the epimyocardium of the heart (Fig. 6H). Expression of *Col2a1* was transient and was absent by 14.5 dpc. However, weak expression of *Sox9* mRNA persisted in the endocardial cushion of the heart as late as 16.5 dpc.

DISCUSSION

The human and mouse *SOX9*/*Sox9* genes encode highly conserved proteins with features of transcriptional regulators, including potential DNA binding and transcriptional activation domains (Foster *et al.*, 1994; Wagner *et al.*, 1994; Wright *et al.*, 1995). The ability to activate transcription has been demonstrated for the mouse proteins SRY, SOX2, SOX4, SOX17, and SOX18 (van de Wetering *et al.*, 1993; Dubin and Ostrer, 1994; Hosking *et al.*, 1995; Kamachi *et al.*, 1995; Kanai *et al.*, 1996), but it is not yet known whether this is a general feature of SOX proteins. We have demonstrated that mouse SOX9 protein can activate transcription of a reporter gene in mammalian cells. Unexpectedly, the strongest transactivation domain for SOX9 was located in the C-terminal 83 amino acid residues and not in the proline- and glutamine-rich domain typical of several known transcription factors. Our data confirm and extend those of Südbeck *et al.* (1996) who demonstrated that the carboxy-terminal 107 amino acids of human SOX9 can activate transcription. As the human and mouse SOX9 proteins show an overall amino acid identity of 96%, it is assumed that the two proteins are biochemically and functionally indistinguishable. It will be of interest to delimit further the region required for transcriptional activation. In this regard

FIG. 6. Expression of *Sox9* and *Col2a1* in nonchondrogenic tissues. *Sox9* expression is shown in A, C, E, and G (left-hand side picture of each pair) and *Col2a1* in B, D, F, and H (right-hand side pictures). (A, B) *Sox9* transcripts are localized to the dorsal region of the neural tube (nt) of a 9.5 dpc embryo and in the sclerotome of the somites (sm), whereas *Col2a1* expression is low in the neural tube but strong in the sclerotome. The notochord (nd) expresses both genes. (C, D) Coexpression in the otic vesicle (ov) at 9.5 dpc. Note the relatively weaker expression of *Sox9* in the paraxial mesoderm (pm). (E, F) Coexpression of both genes in the ventricular neuroepithelium (vne) of the hindbrain and the roof plate (arrows) of an 11.5 dpc embryo. IV marks the fourth ventricle of the hindbrain. (G, H) Differential expression of *Sox9* and *Col2a1* in the 11.5 dpc heart. *Sox9* is expressed in the endocardium (arrowhead) of the ventricle and in the endocardial cushions (ec) flanking the atrioventricular canals (av). *Col2a1*, in contrast, is expressed in the epimyocardium (epm) of the ventricular and the interatrial region. Strong expression of both genes is found in the pharyngeal mesenchyme (phm) but *Col2a1* is preferentially expressed in the pharyngeal pouch (pp) and the arch mesenchyme adjacent to the arch artery (aa). Bar = 50 μm (A, B, G, H) and 100 μm (C–F).

TABLE 2
Differential Expression of *Sox9* and *Col2a1* in Nonskeletal Tissues

Tissues	*Sox9*	*Col2a1*
7.5 dpc		
Trophoblast, ectoplacental cone	+	−
Visceral endoderm	−	Over cardiogenic mesoderm
Gut		
Foregut portal endoderm	Weak, patchy	+
Hindgut endoderm	Weak, patchy	−
Oesophagus		
Epithelium	+	−
Mesenchyme	+	−
Hepatic diverticulum, septum transversum	+	Patchy
Pancreas		
Ductal epithelium	+ (14.5, 15.5 dpc)	−
Islet	−	+ (14.5 dpc)
Intestine		
Mucosa	+ (11.5–16.5 dpc)	−
Submucosa	+ (16.5 dpc)	+
Root of mesentery	+	−
Branchial arches		
I and II arch mesenchyme	− (Later in maxillary prominence)	Weak
III–VI arch	+	−
Pharynx		
Endoderm	−	+
Surface ectoderm	+	+
Lower lip		
Mesenchyme	−	+
Alveolar ridge mesenchyme	−	+
Tongue mesoderm	−	+ (distal tip)
Submandibular gland (duct)	−	+
Respiratory tract		
Bronchial epithelium	+	+
Lung bud mesenchyme	+	−
Limb bud		
Surface ectoderm	Weak	Strong
Mesenchyme	Preaxial > postaxial	Restricted to condensed mass
Urogenital structures[a]		
Genital ridge mesenchyme	+ (12.5 dpc)	−
Metanephric tubules	+ (12.5 dpc)	±
Genital tubercle mesenchyme	+ (14.5 dpc)	−
Others		
Tail tendon	−	+
Cornea	−	+ (17.54 dpc)
Umbilical cord (membrane)	+ (11.5, 12.5 dpc)	−
Heart		
Epimyocardium	−	+
Endocardium	+	−

[a] Sex unknown.

it may be significant that the terminal 20 amino acids of SOX9 from mouse (Wright *et al.*, 1995), human (Foster *et al.*, 1994; Wagner *et al.*, 1994), chicken (GenBank Accession No. U12533), and rainbow trout (N. Takamatsu, personal communication) show a higher degree of conservation than the remainder of the terminal 83-amino-acid domain. Further, the terminal 20 amino acids of mouse and human SOX9 show 60% identity to the rainbow trout SOX8 sequence (Ito *et al.*, 1995), whereas the remainder of the 83 amino acid region is only 18% identical. Many of the *SOX9* mutations in CD patients have been found to affect the composition of the HMG box or to lead to severe truncation

or missense translation of the region downstream from the HMG box (Foster *et al.,* 1994; Wagner *et al.,* 1994; Kwok *et al.,* 1995), confirming the importance of both domains for SOX9 function. Indeed, Südbeck *et al.* (1996) have demonstrated that a SOX9 protein with an intact HMG box but lacking an activation domain can act as a dominant negative regulator of transcription. In view of the present results it will be important to determine the effect on *Col2a1* transcription and the developmental consequences of mimicking similar CD mutations in transgenic mice.

While the gross pattern of expression of *Sox9* has been described previously (Wright *et al.,* 1995), a comprehensive survey of the timing and sites of *Sox9* mRNA and protein expression during development has not been reported. The results presented here suggest that, in addition to its major roles in chondrogenesis and skeletal development, *Sox9* is important for a variety of developmental processes, with expression observed in the developing CNS, notochord, lungs, heart, and urogenital system. The functional significance of these sites of expression is confirmed by the observation that *SOX9* mutations in CD patients commonly affect a variety of nonskeletal organs such as the brain, tracheopulmonary system, heart, and urinary tract (Lee *et al.,* 1972; Houston *et al.,* 1983; Rodriguez, 1993) and frequently result in XY sex reversal (Hovmoller *et al.,* 1977; Tommerup *et al.,* 1993). In addition to genes important for skeletal development, those involved in morphogenesis of other tissues affected by CD, and in the testis-determining pathway, must be considered potential targets for the SOX9 protein.

Given that SOX9 can activate transcription and may function as a transcription factor, it is necessary to identify target genes in order to clarify its developmental role. To date, the only SOX protein for which a target has been identified is SOX2. In the mouse, *Sox2* mRNA is expressed in the lens of the embryonic eye, and SOX2 protein was shown to activate the chicken δ1-crystallin gene encoding the major protein component of the lens (Kamachi *et al.,* 1995). To investigate the possibility of a regulatory relationship between SOX9 and *Col2a1,* the gene encoding the major structural component of cartilage, we have directly compared the expression of these two genes in a comprehensive series of adjacent sections of mouse embryos ranging from 7.5 to 18.5 dpc. Coexpression of these genes was extensive in skeletal tissues, as summarized in Fig. 5. The onset of expression of the two genes appeared to be tightly coupled. The precise relative timing of *Sox9* and *Col2a1* expression could not be resolved in all tissues by sampling of embryos at 1-day intervals, but appearance of *Sox9* mRNA was found to precede that of *Col2a1* transcripts in the chondrogenic mesenchyme of the limb and in the notochord. Both SOX9 protein and *Col2a1* mRNA are present in prechondrogenic mesenchyme and proliferating and maturing chondrocytes. *Sox9* and *Col2a1* also continue to be strongly expressed in cartilaginous tissues which do not ossify and where chondrocytes do not hypertrophy, such as the larynx and bronchial cartilages. The temporal and spatial similarity of these expression patterns suggests that SOX9 is a regulator of *Col2a1* in differentiating chondrocytes, either directly or indirectly. The coexpression of SOX9 protein and *Col2a1* mRNA in some nonchondrogenic tissues such as notochord, otic vesicle, and the developing CNS suggests that positive regulation of *Col2a1* by SOX9 may also occur in these other tissues.

Despite the simplicity of a model in which SOX9 directly or indirectly activates transcription of the *Col2a1* gene, several observations indicate that the transcriptional regulation of *Col2a1* is likely to be more complex. First, *Sox9* expression was down-regulated in hypertrophic chondrocytes and at sites of endochondral ossification by 17.5–18.5 dpc, whereas *Col2a1* expression declined to a lesser degree. Second, *Col2a1* expression in mature cartilage persisted beyond that of *Sox9* mRNA and protein during later stages of fetal development, indicating that SOX9 is not required for the longer term maintenance of *Col2a1* expression. Third, the two genes show differential expression in several nonchondrogenic tissues such as the heart and lung. Fourth, transcriptional activation by other HMG proteins such as LEF-1 and SOX-2 requires cooperation of other factors (Carlson *et al.,* 1993; Giese and Grosschedl, 1993; Kamachi *et al.,* 1995; Yuan *et al.,* 1995). These observations, and the known involvement of multiple factor interactions in transcriptional regulation (Tjian and Maniatis, 1994), suggest that SOX9 also requires other tissue-specific cofactors for transactivation of its target genes. The involvement of *Sox9* in unrelated processes such as sex determination (Kent *et al.,* 1996; Morais da Silva *et al.,* 1996) also points to varying combinations of SOX9 and other transcription factors to achieve different regulatory functions. The determination of the precise molecular mechanisms by which SOX9 regulates chondrogenesis and the identification of potential cooperating factors are important goals of future research.

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