A New Long Form of c-Maf Cooperates with Sox9 to Activate the Type II Collagen Gene*

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A new long form of the c-Maf transcription factor (Lc-Maf) was identified and shown to interact specifically with SOX9 in a yeast two-hybrid cDNA library screening. Lc-Maf encodes an extra 10 amino acids at the carboxyl terminus of c-Maf and contains a different 3’-untranslated region compared with c-Maf. The interaction between SOX9 and Lc-Maf was further confirmed by co-immunoprecipitation and glutathione S-transferase pull-down assays, which mapped the interacting domain of SOX9 to the high mobility group box DNA binding domain and that of Lc-Maf to the basic leucine zipper motif. In situ hybridizations showed that Lc-Maf RNA was coexpressed with Sox9 and Col2a1 RNA in areas of precartilaginous mesenchymal condensations during mouse embryo development. A DNA binding site of Lc-Maf was identified at the 5’-end of a 48-bp Col2a1 enhancer element near the high mobility group binding site of SOX9. Lc-Maf and SOX9 synergistically activated a luciferase reporter plasmid containing a Col2a1 enhancer and increased the transcription of the endogenous Col2a1 gene. In summary, Lc-Maf is the first transcription factor shown to interact with Sox9, to be coexpressed with Sox9 during an early step of chondrogenesis and to cooperate with Sox9 in activating a downstream target gene of Sox9.

In the multistep process of chondrogenesis, condensation of mesenchymal cells is the first identifiable morphological change and a pivotal step in chondrocyte differentiation (1). These mesenchymal condensations prefigure the shape of the cartilages that are the models for endochondral bone formation. Whereas the cells in the center of the condensations differentiate into mature chondrocytes, the more peripheral cells form a layer of cells that becomes the perichondrium. In the epiphyseal growth plate of endochondral bones, chondrocytes undergo a unidirectional proliferation that is mainly responsible for the longitudinal growth of bones and then change their genetic program and become hypertrophic. Several cytokines, growth factors, extracellular matrix components, and transcription factors have been shown to play important roles in discrete stages of chondrogenesis (2–4).

Sox9 is a typical transcription factor containing a high mobility group (HMG) box DNA binding domain and a potent transcription activation domain. Heterozygous mutations of SOX9 in humans cause campomelic dysplasia, a disease characterized by hypoplasia of most endochondral bones and often associated with sex reversal (5–9). More importantly, Sox9 null mutant cells in mouse embryo chimeras were excluded from mesenchymal condensations and failed to express Col2a1 and other chondrocyte-specific marker genes such as Col9a2, Col11a2, and aggrecan, indicating that Sox9 is required for mesenchymal condensations and subsequent cartilage formation (10). Heterozygous Sox9 mutant mice phenocopy the skeletal anomalies of patients with campomelic dysplasia. Histological analysis of these heterozygous mutant mice indicated that Sox9 haploinsufficiency results in defective cartilage primordia and premature skeletal mineralization. These results suggested the hypothesis that both mesenchymal condensations of cartilage primordia and the rate of transition of chondrocytes into hypertrophic chondrocytes were sensitive to Sox9 dosage (11). Recently, we also showed that phosphorylation of SOX9 by protein kinase A increased its DNA binding and transcriptional activities (12). The levels of phosphorylated Sox9 in the growth plate were shown to be highest in the prehypertrophic chondrocytes, the same area where parathyroid hormone/parathyroid hormone-related protein receptor is expressed at high levels. Because parathyroid hormone/parathyroid hormone-related protein also increased the transcriptional activity of SOX9 in DNA transfection experiments, it was suggested that Sox9 may mediate some of the effects of parathyroid hormone-related protein in regulating the transition from proliferating chondrocytes into hypertrophic chondrocytes (13). Sox9 binds to specific sequences in enhancers or promoters of several chondrocyte-specific genes and activates these DNA segments in reporter constructions (14). Moreover, ectopically expressed SOX9 activates one of these genes, the endogenous Col2a1 gene, in some noncartilaginous sites in transgenic mice (15). Sox9 and Col2a1 are coexpressed in all chondrocyte precursors and chondrocytes but are not expressed in hypertrophic chondrocytes (16, 17).

Two other members of the Sox family, a new long form of Sox5 (L-Sox5) and Sox6, were identified in chondrocytes and shown to be coexpressed with Sox9 during chondrogenesis (18). The three Sox proteins cooperated to activate expression of the Col2a1 and aggrecan genes in vitro (18). Whereas Sox5- and Sox6-null mutants had only mild skeletal abnormalities, Sox5, Sox6 double null mutants showed a very severe chondro-
dysplasia characterized by an almost complete absence of cartilage (19).

To identify proteins that interact with Sox9 during chondrogenesis, we used a yeast two-hybrid method consisting of a modified son of sevenless (SOS) recruitment system (20) to screen a chondrocyte cDNA library. One of the cDNAs coded for a new long form of c-Maf (Lc-Maf) that interacted specifically with Sox9. c-Maf is a proto-oncoprotein with a basic leucine zipper (bZip) motif (21) and belongs to a distinct subgroup of the bZip family of transcription factors (22). c-Maf and related proteins such as MafB and NF-AT play important roles in several developmental and cell differentiation processes (23). c-Maf forms both homodimers and heterodimers with other Maf family members and with AP-1 family proteins (24). It also interacts with other transcription factors that either enhance (25) or inhibit its transcriptional activity (26, 27). Although a Maf consensus DNA binding sequence was identified by in vitro site selection (28), interaction of c-Maf with other proteins usually changes its binding specificity, hence producing a diversity of regulatory elements in different target genes. We show here that Lc-Maf, which has an extra 10 amino acids at the carboxyl terminus of c-Maf, interacted directly with Sox9. Le-Maf and Sox9 were co-expressed during mesenchymal condensations, synergistically activated a Col2a1 chondrocyte-specific enhancer, and increased expression of the endogenous Col2a1 gene. Our results are consistent with the hypothesis that Le-Maf and its interaction with Sox9 may play an important role during mesenchymal condensations, a critical step in chondrogenesis.

EXPERIMENTAL PROCEDURES

cDNA Library Construction and SOS Recruitment System Screening—The construction of a primary chondrocyte cDNA library and SOS recruitment system library screening were performed as described previously (12). One of the positive clones, which encoded the full-length Le-Maf cDNA, was cotransfected with either the empty vector or pADNS-cjun-SOS or pADNS-SOS-Sox9 plasmid. The cotransfected cells were plated on glucose or galactose plates to test the specificity of interactions as described previously (12).

RNA Analysis—Total RNA extraction and Northern hybridization were performed as described previously (14). The Le-Maf probe used was a 700-bp fragment cleaved by ApaI and EcoRI from the 3'-untranslated region (UTR) of a cDNA clone encoding the full-length Lc-Maf cDNA. Total RNA was extracted by using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Fifteen micrograms of each RNA sample was electrophoresed, and Northern hybridization with a Col2a1-specific probe was performed as described previously (14). The blot was stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase probe (Ambion, Austin, TX) as loading control.

In Vitro Transcription-Translation—Full-length and deleted constructs of Sox9 and Le-Maf were labeled with [35S]methionine and generated by in vitro transcription-translation with the single-tube TNT protein system (Novagen, Madison, WI) according to the manufacturer’s instructions.

Co-immunoprecipitation—COS-7 cells were cotransfected with pcDNA3.1 expression plasmids for Sox9 and Lc-Maf containing a FLAG tag sequence at its N terminus. Twenty-five microliters of cell lysates was incubated with 15 µl of affinity-purified Sox9 antibody, Sox9 preimmune serum, or c-Maf antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), which also recognizes Le-Maf, in phosphate-buffered saline containing 0.5% Nonidet P-40 for 4 h at 4°C. Then 2.5 µl of protein A-Sepharose 4B (Sigma) resin was added in a final volume of 50 µl. After incubation at 4°C for another 2 h, the resin was washed three times with 1 ml of incubation buffer. The resin was then boiled in SDS-PAGE loading buffer, electrophoresed in an SDS-10% polyacrylamide gel, and transferred to a nylon membrane. Western blotting was performed as described previously (12) with either mouse FLAG m2 (Sigma) or rabbit Sox9 antibody diluted 1:100 followed by HRP-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL).

GST Pull-down Assay—GST Sox9 and GST Le-Maf were expressed and purified according to the method described previously (12). Two microfilters of [35S]-labeled wild-type Sox9 or Sox9 deletions was incubated with 500 ng of either GST or GST-Le-Maf in an interaction buffer containing 25 mM HEPES, pH 7.9, 100 mM NaCl, 1 mM EDTA, 0.5 mM Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol for 30 min at room temperature; 20 µl of glutathione-agarose resin (Sigma) that had been washed three times in interaction buffer was then added in a total volume of 100 µl. After incubation at 4°C for another 30 min, the resin was washed three times with 500 µl of the incubation buffer. The resin was then boiled in 10 µl of SDS-PAGE loading buffer and run on an SDS-10% or 15% polyacrylamide gel, which was then dried and autoradiographed. A parallel SDS-polyacrylamide gel loaded directly with 2 µl of each labeled protein sample was run as the loading control and analyzed in the same manner. 35S-labeled wild-type Le-Maf and Le-Maf deletions with GST or GST-SOx9 were incubated in the same manner.

Electrophoretic Mobility Shift Assays—The R2 18-bp Col2a1 enhancer probe was described previously (14). Wild-type and mutant R1 20-bp probes containing 2-bp mutations were prepared by annealing 20-mer oligonucleotides that were then 5'-end 32P-labeled. The 32P-labeled probes were then incubated with 2–10 ng of nuclear extract prepared from mouse liver or NIH 3T3 cells. The reactions were electrophoresed on a 5% polyacrylamide gel, which was then dried and autoradiographed. The 32P-labeled probes were visualized by chemiluminescence (Amersham) and scanned with a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

FIG. 1. Interaction of Le-Maf with Sox9 in a yeast two-hybrid assay. A, specific interaction between Sox9 and Le-Maf. A plasmid isolated from a positive colony by SOS recruitment system library screening, which encodes the full-length Le-Maf cDNA, was retransformed into a cdc25–2 temperature-sensitive mutant yeast strain together with either empty pADNS, pADNS-cjun-SOS, or pADNS-SOS-Sox9. Four independent colonies generated from each plasmid combination were replica-plated onto galactose and glucose plates and grown at 37°C for 4 days. The expression of Le-Maf was under the control of a GAL-1 promoter, and only the colonies cotransformed with SOS-Sox9 and Le-Maf grew on the galactose plates but not on the glucose plate. B, alignment of c-Maf and Le-Maf proteins, showing functional domains. LLLLLV represents the bZip motif. Le-Maf had an extra 10 amino acids at the carboxyl terminus of c-Maf and a completely different 3’-UTR. C, alignment of the last 10 C-terminal amino acids of Le-Maf (mouse), a long form of c-Maf (chicken), and V-Maf. Vertical lines show identical amino acids.
between the described previously (18). All deletions were inserted into pcDNA 3.1 C9-304, N9-199, N9-HMG, N9-104) or by a two-step PCR (F9^PQA) as other SOX9 deletions were generated either by one-step PCR (N9-400, full-length SOX9 was cloned into GEX4T3 (Amersham Biosciences). All were cloned into pcDNA 3.1 as described previously (12, 14). Similarly, mouse embryo sections at different developmental stages was per-

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   :alt: Image of mouse embryo sections

formed as described previously (18). The cells were then transfected with luciferase reporter plasmids containing an 89-bp Co2a1I promoter without (p89Luc) or with four copies of a 48-bp (4×48-p89Luc) or two copies of a 100-bp (2×100-p88Luc) Co2a1I enhancer element and the plasmid pSV2-β-gal as an internal control for transfection efficiency. The reporter plasmids and pSV2-β-gal were transfected in a ratio of 3:1 as described previously (12). Expression plasmids for SOX9 (100 ng) and different c-Maf plasmids (including c-Maf, Le-Maf, and the Le-maf deletion mutant, Le-Maf^TA, each 100 ng) were transfected by using FuGene6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Luciferase and β-galactosidase activities were assayed in cell lysates prepared as described previously (12). Reporter activities were reported as the average of triplicate cultures in one of several representative experiments as previously described (12).

Interaction of Lc-Maf with SOX9 in a Yeast Two-hybrid Assay—Using the full-length human SOX9 as bait, we screened a primary chondrocyte cDNA library by the using SOS recruitment system as described previously (12). We obtained two independent cDNA clones encoding c-Maf. One of these contained the entire coding sequence of a new isoform of c-Maf, designated here as Le-Maf. We used this clone to test specific interaction with Sox9 (Fig. 1A). Rescue of the growth of cdc25-2 Saccharomyces cerevisiae cells on galactose at high temperature only occurred upon cotransformation of both SOS-SOX9 and Le-Maf plasmids, indicating that there is a specific interaction between Le-Maf and SOX9. The Le-Maf cDNA had 5’-untranslated and coding regions identical to the previously identified mouse c-Maf (21) but had an extra 10 amino acids at the carboxyl terminus and contained a completely different 3’-UTR (Fig. 1B). Protein alignment of the last 10 amino acids at the carboxyl terminus of Le-Maf with those of a similar long form of chicken c-Maf (GenBank™ D28589) and v-Maf (29; Fig. 1C) revealed that they were highly conserved. c-Maf and Le-Maf probably result from differential splicing of a single gene.

RNA Expression of Le-Maf—To investigate the RNA expression pattern of Le-Maf, we used a 700-bp fragment from the 3’-UTR of Le-Maf as a probe and performed a Northern hybridization of RNA from various cell lines, including chondrocytes, and various tissues from newborn mice (Fig. 2). The 4.5-kb Le-Maf RNA was expressed strongly in primary chondrocytes, less in MC615 mouse immortalized chondrocytes, and even less..
Le-Maf and Sox9 in Type II Collagen Activation

Fig. 4. GST pull-down assays mapping the interacting domains of SOX9 and Le-Maf. A, different deletions of SOX9 generated either by PCR or by direct enzyme digestion and then cloned into pcDNA 3.1. Proteins were synthesized in vitro from these constructs and labeled with [35S]methionine. The names of each protein were based on the positions and numbers of amino acids being deleted. B, the HMG-box DNA binding domain of SOX9 interacted with GST-Le-Maf. Labeled SOX9 and SOX9 deletions were incubated with either GST alone (lane 0) or GST-Le-Maf (lanes 1–8). Shown are autoradiographs made after separation by 10% (upper panels) or 15% (lower panels) SDS-PAGE. Parallel gels with loading controls for the same proteins are shown in the left panels, and those with pulled down proteins are shown in the right panels. C, deletions of Lc-Maf. The various Le-Maf deletions were expressed and labeled in the same manner as described for SOX9 in B. D, the bZip motif of Le-Maf interacted with GST-SOX9. The assay was performed, and the results were analyzed as described for B.

in 10T1/2 fibroblasts, which express low levels of Sox9 and Col2a1. Le-Maf transcripts were also present in EL4 lymphoma cells. Le-Maf RNA was highly expressed in brain, skin, intestine, and kidney but at only very low levels or not at all in spleen, heart, testis, and tongue. Therefore, although Le-Maf was widely expressed, it is not a ubiquitous factor.

Co-immunoprecipitation of Le-Maf with SOX9—To confirm the interaction between Le-Maf and SOX9, we cotransfected COS-7 cells with Sox9 and an Le-Maf expression plasmid containing an in-frame FLAG tag sequence at its N terminus and performed a co-immunoprecipitation. As shown in Fig. 3, A and B, Le-Maf and SOX9 were efficiently expressed in COS-7 cells. Lysates of transfected cells were immunoprecipitated with a SOX9 antibody, and the precipitates were analyzed by Western blotting with an anti-FLAG m2 antibody. As shown in Fig. 3C, only the cell lysates cotransfected with SOX9 and Le-Maf contained an immunoprecipitated polypeptide the size of FLAG-Le-Maf. Next, we tested whether c-Maf antibody could also co-immunoprecipitate SOX9 in transfected COS-7 cell lysates. As shown in Fig. 3D, SOX9 was co-immunoprecipitated with c-Maf antibody but not with Sox5 antibody.

Interaction of the bZip Motif of Le-Maf with the HMG DNA Binding Domain of SOX9—To identify the interacting domains in SOX9 and Le-Maf, we generated various deletions of Sox9 (Fig. 4A) and Le-Maf (Fig. 4C). All deletions were labeled with [35S]methionine by using an in vitro transcription-translation reaction. A GST pull-down assay was performed by incubating purified GST or GST-Le-Maf with various deletions of SOX9. GST did not pull down either full-length SOX9 (Fig. 4B, lane 0) or any SOX9 deletions (data not shown). All SOX9 constructions containing the HMG-box DNA binding domain were pulled down by GST-Le-Maf (Fig. 4B, lanes 1–4 and 6). A deletion construct containing the N terminus of SOX9 (lane 8) and a deletion containing the carboxyl-terminal transactivation domain of SOX9 (lane 5) could not be pulled down, indicating that the HMG-box DNA binding domain of SOX9 mediated the interaction with Le-Maf. Likewise, GST-SOX9 pulled down both the full-length Le-Maf and truncated Le-Maf without the N-terminal activation domain or a small segment of Le-Maf containing the bZip motif (Fig. 4D, lanes 1, 2, and 4). In contrast, a truncated Le-Maf lacking only the carboxyl-terminal part of the bZip domain was not pulled down by GST-SOX9 (Fig. 4D, lane 3), indicating that the bZip domain of Le-Maf is the interacting motif.

Binding of Le-Maf to a GC-rich Sequence at the 5’-End of the 48-bp Col2a1 Enhancer Element—Previously SOX9 was shown to bind strongly to an HMG-related sequence at the 3’-end of the 48-bp Col2a1 enhancer element (18). However, no binding of Le-Maf was observed by using an 18-bp segment at the 3’-end of this 48-bp segment (R2) to which SOX9 binds strongly (Fig. 5B). In contrast, Le-Maf bound to a 20-bp fragment containing the 5’ region of the 48-bp element (R1) (Fig. 5B). The sequence GGCTCTG, which is only 1 nucleotide different from the sequence GGCTCAG, which was previously found to be a binding site for c-Maf in the L7 Purkinje neuron-specific promoter (21), was present in this 20-bp Col2a1 segment. To narrow down the Le-Maf binding sequence in the 48-bp element, we generated a series of 2-bp mutations shown in Fig. 5A. These mutated R1 probes (M5, M6, and M7), bound Le-Maf...
activation of the Maf and L-Sox5 (data not shown). The specificity of Lc-Maf out the transcription activation domain (Fig. 6A). Coexpression of L-Maf and SOX9 produced more than an additive activation of the 48-bp Col2a1 enhancer (Fig. 6A). This synergism was also observed with the short and long forms of c-Maf (Lc-Maf). Lc-Maf had 10 extra amino acids at the carboxyl terminus and a completely different 3′ untranslated region. A similar long form of c-Maf (Lc-Maf) was identified in chickens and second branchial arches (Fig. 8A). In E13.5 embryos, expression of Lc-Maf was strong in the perichondrium, whereas Sox9 was strongly expressed in the center of the condensations, where cells undergo overt chondrocyte differentiation (Fig. 8D). This suggests that Lc-Maf and the interaction between Lc-Maf and Sox9 may play a role in chondrogenesis, mainly during mesenchymal condensations.

**DISCUSSION**

Using a modified yeast two-hybrid system to screen for polypeptides interacting with SOX9, we identified a new long form of c-Maf (Lc-Maf). Lc-Maf had 10 extra amino acids at the carboxyl terminus and a completely different 3′-untranslated region. A similar long form of c-Maf was identified in chickens as a differential splicing product (GenBankTM D28598), suggesting that the mouse Lc-Maf may be derived by the same mechanism. The functional difference between the short and long forms of c-Maf is still unknown. Very similar Northern and *in situ* hybridizations with sections from mouse embryos at various developmental stages by using probes specific for Lc-Maf, Sox9, and Col2a1. As shown in Fig. 8, Lc-Maf, Sox9, and Col2a1 were coexpressed in cartilaginous primordia during mesenchymal condensation between embryonic days 10.5 and 12.5 (E10.5 and E12.5), including the first and second branchial arches (Fig. 8A), forelimb and hind limb buds (Fig. 8A), vertebrae (Fig. 8B), and Meckel’s cartilage and nose (Fig. 8C). In E13.5 embryos, expression of Lc-Maf was strong in the perichondrium, whereas Sox9 was strongly expressed in the center of the condensations, where cells undergo overt chondrocyte differentiation (Fig. 8D). This suggests that Lc-Maf and the interaction between Lc-Maf and Sox9 may play a role in chondrogenesis, mainly during mesenchymal condensations.

**Coexpression of Lc-Maf with Sox9 and Col2a1 Genes during Mesenchymal Condensation**—To better understand the physiological significance of the interactions between SOX9 and Lc-Maf, we performed *in situ* hybridizations with sections from mouse embryos at various developmental stages by using probes specific for Lc-Maf, Sox9, and Col2a1. As shown in Fig. 8, Lc-Maf, Sox9, and Col2a1 were coexpressed in cartilaginous primordia during mesenchymal condensation between embryonic days 10.5 and 12.5 (E10.5 and E12.5), including the first and second branchial arches (Fig. 8A), forelimb and hind limb buds (Fig. 8A), vertebrae (Fig. 8B), and Meckel’s cartilage and nose (Fig. 8C). In E13.5 embryos, expression of Lc-Maf was strong in the perichondrium, whereas Sox9 was strongly expressed in the center of the condensations, where cells undergo overt chondrocyte differentiation (Fig. 8D). This suggests that Lc-Maf and the interaction between Lc-Maf and Sox9 may play a role in chondrogenesis, mainly during mesenchymal condensations.

**Synergistic Activation of a Col2a1 Chondrocyte-specific Enhancer by Lc-Maf and SOX9**—To test the functional relevance of the interaction between Lc-Maf and SOX9, we cotransfected the of Lc-Maf and SOX9 expression plasmids into COS-7 cells to test whether they cooperated in activating reporter plasmids containing four copies of a 48-bp or two copies of a 100-bp Col2a1 chondrocyte-specific enhancer (12). We have observed previously that SOX9 strongly activates the 4 × 48-bp Col2a1 reporter (12). In comparison, the effect of Lc-Maf was more moderate (Fig. 6A). Coexpression of Lc-Maf and SOX9 produced more than an additive activation of the 4 × 48-bp Col2a1 enhancer (Fig. 6A). This synergism was also observed with the 2 × 100-bp Col2a1 enhancer, which Lc-Maf activated as strongly as SOX9 did, suggesting that Lc-Maf itself is a strong activator of this Col2a1-specific enhancer (Fig. 6B). Cotransfection of Lc-Maf and SOX9 produced 3 times more activation than did each plasmid alone. This joint activation was not observed with SOX9 and Lc-Maf^TA, a truncated Lc-Maf without the transcription activation domain (Fig. 6A), or with Lc-Maf and L-Sox5 (data not shown). The specificity of Lc-Maf activation of the Col2a1 enhancer was further demonstrated by the lack of response of M7, a mutant 48-bp Col2a1 enhancer containing a 2-bp point mutation in the Lc-Maf binding site (Fig. 6C). As shown in Fig. 6B, M7 decreased the binding of Lc-Maf. Moreover, with mutant enhancers containing either a 2-bp mutation located between the Lc-Maf and SOX9 binding sites or a 2-bp mutation in the SOX9 binding site, activation by Lc-Maf was unchanged. These experiments suggested that DNA binding was required for the activation by Lc-Maf of this Col2a1 enhancer. These results indicated that Lc-Maf was a potent activator of a Col2a1-specific enhancer and that Lc-Maf cooperated with SOX9 to activate this enhancer more strongly.

**Increased Endogenous Col2a1 RNA Expression by Cotransfection of SOX9 and Lc-Maf Expression Plasmids**—We next tested whether coexpression of Lc-Maf and SOX9 increased transcription of the endogenous Col2a1 gene. Transfection of either SOX9 (Fig. 7, lanes 2) or Lc-Maf (Fig. 7, lanes 3) expression plasmids alone into 10T1/2 cells, which express low levels of Col2a1, produced almost no increase of this transcript. However, cotransfection of Lc-Maf and SOX9 produced significantly more Col2a1 RNA (Fig. 7, lanes 4), suggesting that Lc-Maf and SOX9 cooperate, in accord with the previous transient transfection experiments. Similar results were obtained with MC615 cells (Fig. 7, lanes 5–8), in which expression of the endogenous Col2a1 is low after repeated passages in culture. These results strongly suggest that Lc-Maf and SOX9 cooperatively activate the endogenous Col2a1 gene.

**Fig. 5.** Lc-Maf binding to a GC-rich sequence at the 5′-end of a 48-bp Col2a1 enhancer element. A, sequence alignment of a 48-bp enhancer element with wild type and different 2-bp point mutations of 20-bp R1 probe as well as the 18-bp R2 probe. The 2-bp point mutations introduced into R1 are indicated by boldface letters. The SOX9 binding sequence in R2 is boxed, as is a putative GC rich Lc-Maf binding sequence in the 48-bp enhancer element. B, Lc-Maf binding to a 5′ GC-rich sequence (GGCTCTG) in the 20-bp R1 fragment of the 48-bp enhancer but not the 3′ 18-bp R2 fragment. Lc-Maf was expressed by *in vitro* transcription and translation reaction, and the probes (R1 and R2) were labeled with [32P]dCTP. All labeled probes were adjusted with unlabeled probe to achieve identical specific activities. A protein mixture from an *in vitro* transcription-translation reaction using the empty vector pcDNA as template indicated by minus sign was used as a control.
direct interaction between SOX9 and Lc-Maf but also mapped the interacting segments to the HMG-box DNA binding domain of SOX9 and the bZip motif of Lc-Maf. These domains were also found to be used in the interactions of these proteins with other transcription factors. For example, the HMG DNA binding domain of SOX9 interacts with SF-1 (29), and the bZip motif of MafB interacts with the DNA binding domain of Ets-1 (30). The Lc-Maf binding sequence in the 48-bp Col2a1 enhancer element is not the typical Maf recognition element, which was determined by in vitro site selection (27). We have not yet determined whether interaction of Lc-Maf with Sox9 and perhaps other proteins increases its affinity for the 48-bp Col2a1 enhancer. Although Lc-Maf alone efficiently activated a Col2a1 chondrocyte-specific enhancer in transient transfection experiments, cotransfection of Lc-Maf with SOX9 resulted in activation that was more than additive, suggesting that there is a synergistic cooperation between these two factors. This was further confirmed in a Northern hybridization experiment showing an increased expression of endogenous Col2a1 when expression vectors for these two factors were cotransfected. However, forced expression of Lc-Maf and SOX9 in other fibroblasts in which the Col2a1 gene was silent failed to induce Col2a1 expression. It is possible that in these cells other factors or coactivators may be needed to switch on the Col2a1 gene.

To investigate the physiological relevance of the interaction between Lc-Maf and Sox9, we performed a detailed in situ hybridization using mouse embryo sections from different developmental stages. During chondrogenesis, Le-Maf was coexpressed with Sox9 and Col2a1 in all areas of mesenchymal condensations, but after E13.5, when overt differentiation of chondrocytes occurs, expression of Lc-Maf was restricted to the perichondrium. We conclude that Lc-Maf and Sox9 may interact during chondrogenic mesenchymal condensation. After condensation, when cells overtly differentiate into chondrocytes, Lc-Maf expression was shut off, whereas the levels of Sox9, L-Sox5, and Sox6 remained high or increased. It should be noted that the studies that showed interactions between Lc-Maf and Sox9 in Type II Collagen Activation

"FIG. 6. Synergistic activation of a Col2a1 chondrocyte-specific enhancer element by Le-Maf and SOX9. A, coactivation of a 4 × 48-bp Col2a1 enhancer by Le-Maf and SOX9. COS-7 cells were transfected with empty vector (lane 1), Le-Maf (lane 2), Le-Maf^TA (lane 3), SOX9 (lane 4), and SOX9 plus Le-Maf (lane 5) or Le-Maf^TA (lane 6). B, coactivation of a 2 × 100-bp Col2a1 enhancer by Le-Maf and SOX9. COS-7 cells were transfected with empty vector (lane 1), c-Maf (lane 2), Le-Maf (lane 3), SOX9 (lane 4), and SOX9 plus c-Maf (lane 5) or Le-Maf (lane 6). C, abolishment of the Le-Maf activation of a 48-bp enhancer containing a 2-bp mutation in the Le-Maf binding site. The wild type and three different mutated 48-bp enhancer sequences are shown at the left panel. COS-7 cells were transfected with 100 ng of Le-Maf expression plasmid and a luciferase reporter plasmid containing either no enhancer element (lane 1) or different 48-bp enhancer elements indicated (lanes 2–5)."

"FIG. 7. Increased expression of endogenous Col2a1 gene by cotransfection of Le-Maf and SOX9 expression plasmids. 10T1/2 cells (A) or MC615 cells passaged three times (B) were transfected with the empty vector pcDNA (lanes 1 and 5) or expression plasmids for SOX9 (lanes 2 and 6), Le-Maf (lanes 3 and 7), and SOX9 plus Le-Maf (lanes 4 and 8). The cells were cultured for 36 h after transfection, and RNA was extracted for analysis by Northern hybridization with a Col2a1-specific probe. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH)-specific hybridization was used as the RNA loading control, and a Col2a1 RNA sample from RCS cells that had been hybridized in the same manner was used as a control (left panel)."

"Lc-Maf binding sequence in the 48-bp Col2a1 enhancer element is not the typical Maf recognition element, which was determined by in vitro site selection (27). We have not yet determined whether interaction of Le-Maf with Sox9 and perhaps other proteins increases its affinity for the 48-bp Col2a1 enhancer. Although Le-Maf alone efficiently activated a Col2a1 chondrocyte-specific enhancer in transient transfection experiments, cotransfection of Le-Maf with SOX9 resulted in activation that was more than additive, suggesting that there is a synergistic cooperation between these two factors. This was further confirmed in a Northern hybridization experiment showing an increased expression of endogenous Col2a1 when expression vectors for these two factors were cotransfected. However, forced expression of Le-Maf and SOX9 in other fibroblasts in which the Col2a1 gene was silent failed to induce Col2a1 expression. It is possible that in these cells other factors or coactivators may be needed to switch on the Col2a1 gene.

To investigate the physiological relevance of the interaction between Le-Maf and Sox9, we performed a detailed in situ hybridization using mouse embryo sections from different developmental stages. During chondrogenesis, Le-Maf was coexpressed with Sox9 and Col2a1 in all areas of mesenchymal condensations, but after E13.5, when overt differentiation of chondrocytes occurs, expression of Le-Maf was restricted to the perichondrium. We conclude that Le-Maf and Sox9 may interact during chondrogenic mesenchymal condensation. After condensation, when cells overtly differentiate into chondrocytes, Le-Maf expression was shut off, whereas the levels of Sox9, L-Sox5, and Sox6 remained high or increased. It should be noted that the studies that showed interactions between Le-Maf and Sox9 in Type II Collagen Activation were performed in COS-7 cells, which are not the..."
chondrogenic mesenchymal condensations occur normally, but cells in the condensations are unable to differentiate into chondrocytes and to express the high level of cartilage extracellular matrix genes that are characteristic of chondrocytes (19). In addition to the Sox9 binding site, the 48-bp Col2a1 enhancer contains three HMG-box binding sites to which L-Sox5 and Sox6 presumably bind. Mutations in two of these sites outside the Sox9 binding site abolished enhancer activity in vitro. The Le-Maf binding site overlaps with one of these additional HMG binding sites but not with that for Sox9 (18). The levels of L-Sox5 and Sox6 are relatively lower during mesenchymal condensations than at later stages of chondrogenesis. A binding competition assay showed that Le-Maf competes with L-Sox5 to bind the 48-bp Col2a1 enhancer element (data not shown). Because the in situ hybridization results showed co-expression of Le-Maf with Sox9 and Col2a1 RNAs during chondrogenic mesenchymal condensations, we propose that Le-Maf may inhibit binding of L-Sox5 and Sox6 to the Col2a1 enhancer and similar enhancers in other chondrocyte genes during this early stage of chondrogenesis. After condensation, Le-Maf was strongly expressed in the perichondrium but is no longer expressed in chondrocytes, in which L-Sox5, Sox6, and Sox9 are strongly expressed. Thus, Le-Maf, L-Sox5, and Sox6 may function at different steps of chondrogenesis. We hypothesize that Le-Maf cooperates with Sox9 to activate Col2a1 and perhaps other genes needed for chondrogenic mesenchymal condensations, whereas L-Sox5 and Sox6 would function together with Sox9, mainly at a subsequent step during overt chondrocyte differentiation when cartilage extracellular matrix genes are strongly expressed.

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Lc-Maf and Sox9 in Type II Collagen Activation

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