Leukemia/Lymphoma-related Factor, a POZ Domain-containing Transcriptional Repressor, Interacts with Histone Deacetylase-1 and Inhibits Cartilage Oligomeric Matrix Protein Gene Expression and Chondrogenesis*

Mutations in the human cartilage oligomeric matrix protein (COMP) gene have been linked to the development of pseudoachondroplasia and multiple epiphyseal dysplasia. We previously cloned the promoter region of the COMP gene and delineated a minimal negative regulatory element (NRE) that is both necessary and sufficient to repress its promoter (Issack, P. S., Fang, C. H., Leslie, M. P., and Di Cesare, P. E. (2000) J. Orthop. Res. 18, 345–350; Issack, P. S., Liu, C. J., Prajak, L., and Di Cesare, P. E. (2004) J. Orthop. Res. 22, 751–758). In this study, a yeast one-hybrid screen for proteins that associate with the NRE led to the identification of the leukemia/lymphoma-related factor (LRF), a transcriptional repressor that contains a POZ (poxvirus zinc finger) domain, as an NRE-binding protein. LRF bound directly to the NRE both in vitro and in living cells. Nine nucleotides (GAGGGTCCC) in the 30-bp NRE are essential for binding to LRF. LRF showed dose-dependent inhibition of COMP-specific reporter gene activity, and exogenous overexpression of LRF repressed COMP gene expression in both rat chondrosarcoma cells and bone morphogenetic protein-2-treated C3H10T1/2 progenitor cells. In addition, LRF also inhibited bone morphogenetic protein-2-induced chondrogenesis in high density microcarrier cultures of C3H10T1/2 cells, as evidenced by lack of expression of other chondrocytic markers, such as aggrecan and collagen types II, IX, X, and XI, and by Alcian blue staining. LRF associated with histone deacetylase-1 (HDAC1), and experiments utilizing the HDAC inhibitor trichostatin A revealed that LRF-mediated repression requires deacetylase activity. LRF is the first transcription factor found to bind directly to the COMP gene promoter, to recruit HDAC1, and to regulate both COMP gene expression and chondrogenic differentiation.

The differentiation of uncommitted mesenchymal cells into musculoskeletal tissues, including chondrocytes, osteoblasts, tenocytes, and ligament cells, is a fundamental molecular event of both embryonic development and repair of cartilage, ligament, tendon, and bone (1, 2). After commitment to the chondrocyte lineage, mesenchymal cells undergo condensation, cease expression of type I collagen, and differentiate into a chondrocytic phenotype characterized by expression of collagen types II, IX, and XI and the proteoglycan aggrecan (1, 2). During this process, there appears to be transition cells between type I collagen (expressing mesenchymal cells) and type II collagen (expressing chondrocytes) that are characterized by lack of expression of type II collagen and abundant expression of cartilage oligomeric matrix protein (COMP) (3–11). These cells may represent musculoskeletal precursor cells that have the potential subsequently to differentiate into a variety of musculoskeletal cell types; however, little is known about the generation of these potential precursor cells.

The gene for COMP encodes a pentameric non-collagenous matrix protein (3, 9, 10, 12, 13) that is expressed predominantly in articular cartilage (3, 9–11, 14). Mutations in the human COMP gene have been linked to the development of pseudoachondroplasia and multiple epiphyseal dysplasia (15–27), autosomal dominant forms of short-limb dwarfism characterized by short stature, normal facies, epiphyseal abnormalities, and early onset osteoarthritis (reviewed in Refs. 28–30). Accumulating evidence suggests that COMP may function to stabilize the extracellular matrix of articular cartilage by specific cation-dependent interactions with matrix components, including collagen types II and IX and fibronectin (31, 32).

COMP is synthesized by chondrocytes, osteoblasts, tenocytes, and ligament cells, but not by undifferentiated mesenchymal cells (3–11, 33–38). To delineate cis-elements in the COMP promoter necessary for expression in any of these tissues, we cloned the murine COMP promoter and identified cis-elements necessary for expression in the chondrocytic cell line Swarm rat chondrosarcoma (RCS) (35). We have shown that COMP mRNA and protein are expressed in RCS cells, but not in NIH3T3 fibroblasts. A COMP promoter fragment containing ~1.9 kb of 5′-flanking sequence is specifically active in RCS cells. In cell culture experiments, deletion analysis of the

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The abbreviations used are: COMP, cartilage oligomeric matrix protein; RCS, rat chondrosarcoma; NRE, negative regulatory element; LRF, leukemia/lymphoma-related factor; OCZ/F, osteocalcin-derived zinc finger; HDAC, histone deacetylase; aa, amino acids; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; HEK, human embryonic kidney; TSA, trichostatin A; RT, reverse transcription; BMP-2, bone morphogenetic protein-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LBE, LRF-binding element.
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Distal region of the COMP promoter identified a silencer region situated between –1775 and –1716 that specifically binds protein complexes expressed in non-chondrocytic cells, but not in RCS cells. Competition gel shift experiments localized the binding site to within 30 bp (–1775 to –1746; negative regulatory element (NRE)). This site is necessary and sufficient to repress COMP expression in fibroblast cell lines (35, 39).

Yeast one-hybrid screening has proven to be an effective tool to identify DNA-binding proteins (40, 41), including the rat fibronectin gene (42) and Fgf3 promoter (43). To identify proteins that interact with the NRE in the promoter region of the COMP gene, we screened a yeast expression cDNA library using a tandem repeat of four NREs as bait. These experiments identified the leukemia/lymphoma-related factor (LRF) transcriptional repressor as a binding protein at the NRE. LRF is a nuclear protein with an N-terminal POZ (poxvirus zinc finger) domain and a C-terminal Krüppel-like zinc finger DNA-binding domain (44). LRF was found to be the mouse counterpart of human FBI-1 (factor that binds to the HIV-1 inducer of short transcripts) (45–47) and the rat osteoclast-derived zinc finger (OCZF) protein (48), with identical functionally important molecular domains.

Many transcription factors repress transcription by recruiting histone deacetylases (HDACs) to chromatin. HDACs are classified into two groups based on structural and functional similarities. Class I HDACs, including HDAC1, are expressed in the nuclei of cells in most tissues (49). In this study, we discovered that the transcriptional repressor LRF as a novel regulator controlling COMP gene expression and chondrogenesis and demonstrated that HDAC1 is involved in LRF-mediated gene transcription. LRF, which associates with HDAC1, is the first transcription factor found to bind directly to the COMP gene promoter and to regulate COMP gene expression and chondrogenic differentiation.

**Experimental Procedures**

**Plasmid Constructs**—Yeast reporter vectors (pHIS, pHISi-1, and pLacZi; Clontech) were used to generate NRE-specific reporter constructs. Briefly, a synthetic DNA oligomer containing four tandem repeats of the NRE sequence (AGCCTGGGAGAGGGTCCCTGCCCTAGGAAA) was cloned into the EcoRI/XbaI sites of pHISi, pHISi-1, and pLacZi to produce recombinant proteins in bacteria. Cells were harvested by trypsinization, washed with saline, pelleted, resuspended in lysis buffer (10 mM Tris-HCl (pH 8.0), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, proteinase inhibitors, and 0.3% Nonidet P-40). After 5 min on ice, the lysates were centrifuged at 10,000 g for 5 min at 4 °C, and the pellet nuclei were washed with lysis buffer without Nonidet P-40.

**Preparation of Nuclear Extracts**—Nuclear extracts were prepared from C3H10T1/2 cells and RCS cell lines transfected with the pFLAG-LRF expression plasmid. Cells were harvested by trypsinization, washed with saline, pelleted, and resuspended in lysis buffer (10 mM Tris-HCl (pH 8.0), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, proteinase inhibitors, and 0.3% Nonidet P-40). After 5 min on ice, the lysates were centrifuged at 10,000 g for 5 min at 4 °C, and the pellet nuclei were washed with lysis buffer without Nonidet P-40. The nuclear pellet was resuspended in an equal volume of nuclear extraction buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mg/ml bovine serum albumin, 0.5% Nonidet P-40). The protein gels were used for Western blotting with antibodies against LRF and HDAC1.

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35- and 100-mm dishes for expansion in Dulbecco’s modified Eagle’s medium containing hygromycin at 200 μg/ml. Immediately after the 7 days of culture, the COMP expression plasmid

For PCR of the COMP promoter region using the chromatin-immunoprecipitated DNA, one-tenth of the DNA was PCR-amplified using forward primer 5'-GTAGTCAATAGGCCTGG-

200 μl of NaCl and heated to 65 °C for 4 h to reverse the LRF-DNA cross-link. After treatment with EDTA and proteinase K, the supernatant was extracted with phenol/chloroform and precipitated with ethanol.

Luciferase assays were performed using 20 μl of reporter construct (~1925COMPluc, ~1775COMPluc, ~12935COMPluc, ~592COMPluc, or ~4xREpGL2-Promoter) along with 1 μl of pSGVl plasmid (internal control) and various amounts of pFLAG-LRF expression plasmid. To examine whether HDACs are involved in the LRF-mediated repression, various amounts of trichostatin A (TSA) were added to total cell lysates prepared from HEK293 cells transfected with pFLAG-LRF and the empty pFLAG-F4 vector (51).

To characterize anti-LRF serum, 20 μl of cell lysates prepared from micromass cultures of cloned LRF stable lines, control lines, and parental COMP (1:500 dilution), followed by horseradish peroxidase-conjugated secondary antibody (horseradish peroxidase-1:500), preimmune serum (1:500) or anti-LRF antiserum (1:500) for 1 h. After washing, the secondary antibody (horseradish peroxidase-conjugated anti-mouse immunoglobulin for the anti-FLAG probe and anti-rabbit immunoglobulin for preimmune serum and anti-LRF antisera) (1:2000 dilution) was added. Blots were visualized using an enhanced chemiluminescence kit (Amersham Biosciences). To screen the LRF stable cell lines, cell lysates were prepared from cloned LRF stable lines and control lines, and FLAG-tagged LRF levels were examined with the anti-FLAG probe using the procedures described above.

To examine COMP protein expression in the BMP-2-induced chondrogenic differentiation of C3H10T1/2 cells, 25 μg of cell lysates prepared from micromass cultures of cloned LRF stable lines, control lines, and parental C3H10T1/2 cells maintained in Ham’s F-12 medium containing 10% fetal calf serum in the presence of 100 ng/ml recombinant BMP-2 for 7 days were used to perform an immunoblot assay with rabbit polyclonal antisera to COMP (1:500 dilution), followed by horseradish peroxidase-conjugated anti-rabbit IgG (1:1000 dilution).

Magnetic Bead Assay—The magnetic bead assay was performed as described previously (50). Briefly, a 592-nucleotide proximal region (~592 to ~1) and a 400-nucleotide distal region (~1925 to ~1526, containing the NRE and used as a positive control) of the COMP promoter were labeled with biotin using the Promega random-primed labeling kit (Promega, Madison, WI) and incubated with anti-FLAG antibody (Stratagene, La Jolla, CA), anti-LRF or anti-HDAC1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or preimmune serum overnight at 4 °C. The lysate was sonicated to shear DNA to a length between 200 and 1000 bp. The sonicated supernatant was diluted 10-fold with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM Tris-HCl (pH 8.1), and 150 mM NaCl) and incubated with anti-FLAG antibody (Stratagene, La Jolla, CA), anti-LRF or anti-HDAC1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or preimmune serum overnight at 4 °C with rotation. To remove the DNA and A-agarose slurry was pelleted by centrifugation. After extensive washing of the pellet with a series of wash buffers, the pellet was dissolved with 250 μl of elution buffer and centrifuged to remove the agarose.

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Reporter Gene Assay—RCS cells grown to ~50% confluence in 35-mm culture dishes were transfected with 1 μg of reporter construct (~1925COMPluc, ~1775COMPluc, ~12935COMPluc, ~592COMPluc, or ~4xREpGL2-Promoter) along with 1 μl of pSGVl plasmid (internal control) and various amounts of pFLAG-LRF expression plasmid. To examine whether HDACs are involved in the LRF-mediated repression, various amounts of trichostatin A (TSA) were added to total cell lysates prepared from HEK293 cells transfected with pFLAG-LRF and the empty pFLAG-F4 vector (51).

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RNA Preparation and Reverse Transcription (RT)-PCR—Total RNA was prepared from micromass cultures of cloned LRF stable lines, control lines, and parental C3H10T1/2 cells maintained in Ham’s F-12 medium (Invitrogen) containing 10% fetal calf serum in the presence of 100 ng/ml recombinant BMP-2 (Genetics Institute, Cambridge, MA) for 7 days. RNA was purified as described previously (51) using oligo(dT) primers with the SuperScript preamplification system (Invitrogen) following the manufacturer’s instructions. PCR was performed in a volume of 25 μl for 25 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for the primers of target and control sequences for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 5'-GTCTAGAGACGGCGCATCTT-3' and 5'-ACAGAGCTTGCTAAGAAGG-3' for mouse type II collagen, 5'-GGTGAACAGTGGTTTCTTTC-3' and 5'-ATCAAGTGGCCCTCTACT-3' for mouse aggrecan, 5'-TACCTGCTGCGCCGAGAG-3' and 5'-ACCCCTTAGGCGATGGAC-3' to screen the LRF stable cell lines, cell lysates were prepared from cloned LRF stable lines and control lines, and FLAG-tagged LRF levels were examined with the anti-FLAG probe using the procedures described above.

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Acidic Blue Staining—To assess the extent of chondrogenesis, micromass cultures of cloned LRF stable lines, control lines, and parental C3H10T1/2 cells maintained in Ham’s F-12 medium containing 10% fetal calf serum in the presence of 100 ng/ml recombinant BMP-2 for 14 days were fixed in Kahle’s fixative, washed with water, and stained overnight with 1% Alcan blue 8GX (Sigma) (56).

In Vitro GST Pull-down Assay—To examine whether LRF binds to HDAC1 in vitro, glutathione-Sepharose beads (50 μl) preincubated with 0.5 μg of purified GST (serving as a control), GST-HDAC1(1–432), GST-HDAC1(51–452), GST-HDAC1(51–467), GST-HDAC1(51–482) were incubated with in vitro translated LRF expression plasmid, a rabbit reticulocyte translation/transcription system (Promega) in 150 μl of buffer containing 10 mM Tris-HCl (pH 7.9), 10% glycerol, 100 mM KCl, and 0.5 mg/ml bovine serum albumin. The bound proteins were denatured in sample buffer and separated by 12% SDS-PAGE, and protein was detected by Western blotting with affinity-purified anti-LRF antibodies.

Co-immunoprecipitation—Approximately 500 μg of cell lysates prepared from LRF stable lines were incubated with anti-LRF (25 μg/ml) or anti-HDAC1 (20 μg/ml) antibody or with control rabbit IgG (25 μg/ml) for 1 h, followed by incubation overnight with 30 μl of protein A-agarose (Invitrogen) at 4 °C. After washing five times with immunoprecipitation buffer, bound proteins were released by boiling in 20 μl of 2× SDS loading buffer for 3 min (51). Released proteins were examined by Western blotting with anti-HDAC1 antibody, and the signal was detected by Western blotting with affinity-purified anti-LRF antibodies.
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The Yeast One-hybrid System Identifies the Transcriptional Repressor LRF as an NRE-binding Protein—To identify the repressors that bind to the NRE located at –1775 to –1746 in the mouse COMP gene promoter, yeast one-hybrid screening was carried out according to the Matchmaker one-hybrid protocol. For this purpose, four tandem repeats of the NRE sequence (AGCCTGGGAGAGGGTCCCTGCCCTATGGAA) from the COMP gene promoter were ligated into the yeast integrator and reporter vectors pHISi and pLacZi to generate pHISi-4NRE and pLacZi-4NRE, respectively. Each pHISi-4NRE and pLacZi-4NRE reporter construct was linearized and integrated sequentially into the genome of the competent yeast strain YM4271. The resulting yeast cells with integrated pHISi-4NRE and pLacZi-4NRE were used for one-hybrid screening with a mouse embryonic cDNA/VP16 activation domain fusion library. Screening of 2.5 × 10⁶ clones from a mouse embryonic cDNA library generated seven positive clones. Positive colonies were selected on synthetic complete medium lacking histidine and tryptophan and supplemented with 45 mM 3-amino-1,2,4-triazole and transformed into E. coli DH5α. Each cDNA insert was sequenced and analyzed by a BLAST search of the GenBank™/EBI Data Bank. Two of the positive clones were identical and contained a 1793-bp insert. The nucleotide sequences of these clones contain an open reading frame of 1698 bp, with the first ATG surrounded by an appropriate Kozak consensus sequence (57). The predicted open reading frame encodes a protein of 566 amino acid residues that is identical to mouse sequence (57). The predicted open reading frame encodes a protein of 566 amino acid residues that is identical to mouse sequence (57). The predicted open reading frame encodes a protein of 566 amino acid residues that is identical to mouse sequence (57). The predicted open reading frame encodes a protein of 566 amino acid residues that is identical to mouse sequence (57).

RESULTS

Preparation and Characterization of Anti-LRF Antibodies—The GST-LRF-(207–277) fusion protein, encoding a segment (aa 207–277) between the POZ domain and zinc finger motifs of LRF, was expressed in bacteria, purified on a glutathione-Sepharose column; and subjected to preparative scale SDS-PAGE. The major band was excised and used to immunize rabbits (Zymed Custom Antibody). To generate affinity-purified anti-LRF antibodies, the GST- and LRF-binding elements (aa 207–277) were translated in vitro and subjected to SDS-PAGE and detected with affinity-purified anti-LRF antibodies. The arrow indicates LRF; the arrowhead indicates an unknown protein in the rabbit reticulocyte lysate.

Western blotting was performed using in vitro translated LRF and nuclear extracts prepared from C3H10T1/2 cells transfected with the mamma-meric expression plasmid pFLAG-LRF resulted in a specific growth on the culture plates lacking histidine and tryptophan but containing 45 μM 3-amino-1,2,4-triazole (Fig. 1B).

Interactions identified in yeast must be verified using independent procedures because 1) the yeast one-hybrid system may produce false interactions, and 2) true interactions in yeast in which post-translational modifications are absent might be lost in the mammalian cells if post-translational modifications are involved in the associations. We first confirmed the binding of LRF to the NRE in EMSA (Fig. 3). Incubation of the ³²P-labeled NRE probe with the nuclear extracts prepared from RCS cells transfected with the mammalian expression plasmid pFLAG-LRF resulted in a specific interaction.
LRF binds to the NRE of the COMP promoter in vitro (EMSA). Ten micrograms of nuclear extracts (NE) prepared from RCS cells transfected with pFLAG-LRF were mixed in the reaction buffer (20 μl). For competition experiments, a 100-fold excess of wild-type NRE oligodeoxynucleotide was added. After 15 min of incubation, the 32P-labeled NRE probe was added, and the reaction mixture was incubated for an additional 15 min and analyzed by gel electrophoresis. The positions of the supershifted IgG-FLAG-LRF-NRE complex (supershift), the FLAG-LRF-NRE complex (shift), and the free DNA probe (probe) are indicated.

FLAG-LRF-NRE complex (lane 3). The binding of probe to FLAG-LRF in vitro (lane 3) was completely competed by excess unlabeled oligodeoxynucleotide (lane 1). The FLAG-LRF-NRE band was supershifted with antibodies to FLAG (lane 4).

LRF binds to the NRE in the COMP Gene Promoter in Living Cells—To determine whether LRF also binds to the NRE in vivo, we performed ChIP assays, which are important for defining interactions of factors with specific DNA elements in living cells. ChIP was first carried out in HEK293 cells transfected with the COMP-specific reporter construct −1925COMP:Luc. HEK293 cells transfected with −1925COMP:Luc and the expression plasmid pFLAG-LRF were cross-linked by formaldehyde treatment and lysed. Cell lysates were subjected to immunoprecipitation with control IgG (lane 1) or with anti-FLAG (lane 2) or anti-LRF (lane 3) antibody. Purified DNA from the cell lysate (Input DNA; upper panel) and DNA recovered from immunoprecipitation (IP; lower panel) were amplified by PCR. B, endogenous LRF binds to the NRE of the COMP gene. C5H10T1/2 cells treated with formaldehyde were lysed, and DNA was sheared by sonication. Cell lysates were subjected to immunoprecipitation with either preimmune serum (Preimmune; lane 2) or anti-LRF polyclonal antibodies (lane 3). DNA recovered from the immunoprecipitation was amplified by PCR. Input DNA (lane 1) was used as positive control.

performing EMSA again using wild-type and serial mutant NRE probes. All probes were incubated with nuclear extracts prepared from pFLAG-LRF-transfected RCS cells; the binding of LRF to various probes is summarized in Fig. 5A and shown in Fig. 5B. Mutants 1 and 5, in which the first six nucleotides (AGCTGG) and the last six nucleotides (ATGGAA) were mutated, respectively, bound to LRF as strongly as the wild-type NRE probe (Fig. 5B, compare lanes 1, 2, and 6); however, mutants 2 and 3, in which the second six nucleotides (GGAG) and the third six nucleotides (GGAGAC) were altered, respectively, totally abolished the binding of LRF (lanes 3 and 4), clearly demonstrating that these 12 nucleotides (GGAGACGGTCCC) are essential for binding to LRF. Interestingly, mutant 4, in which the fourth six nucleotides (TGCCCT) were replaced, still bound to LRF, but the binding intensity was weaker (lane 5). Twelve nucleotides (GGAGACGGTCCC) identified above in the NRE contain a typical consensus binding site (G(A/G)GGG(T/C)(C/T)(C/T)(C/T)) (59) of FBI-1, a human counterpart of mouse LRF. To confirm whether the flanking sequences of the consensus binding site are involved in the binding of LRF to the NRE, an additional three mutants of NRE (lanes 7–9) were constructed and tested. Mutant 6, in which three 5′-nucleotides (GGA) flanking the “consensus sequence” were mutated, bound to the LRF as well as the wild-type probe, indicating that these three nucleotides are not required for LRF-NRE association. Mutants 7 and 8, in which the first three nucleotides (TGC) and the last three nucleotides (CCT) of six 3′-nucleotides flanking the consensus sequences were changed, respectively, still bound to LRF with reduced affinity, as did mutant 4, in which these six nucleotides were replaced. These data indicate that the identified core DNA-binding site for LRF in vitro is in accordance with the published consensus sequence for FBI-1. It is also possible that the six 3′-nucleotides of this...
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**A** Binding of NRE

|     | Wt | Mut1 | Mut2 | Mut3 | Mut4 | Mut5 | Mut6 | Mut7 | Mut8 |
|-----|----|------|------|------|------|------|------|------|------|
|     | AGCTGGAGAGGGCTGCTGCTGCTATGGAA | ++   | ++   | –    | –    | ++   | ++   | +    | ++   |
| Mut1| gtaactGGAGAGGGCTGCTGCTGCTATGGAA | ++   | –    | –    | –    | –    | ++   | –    | ++   |
| Mut2| AGCTGGTGGTGGGCTGCTGCTGCTATGGAA | –    | –    | –    | –    | –    | ++   | –    | ++   |
| Mut3| AGCTGGAGAGGGCTGCTGCTGCTATGGAA | –    | –    | –    | –    | –    | ++   | –    | ++   |
| Mut4| AGCTGGAGAGGGCTGCTGCTGCTATGGAA | –    | –    | –    | –    | –    | ++   | –    | ++   |
| Mut5| AGCTGGAGAGGGCTGCTGCTGCTATGGAA | –    | –    | –    | –    | –    | ++   | –    | ++   |
| Mut6| AGCTGGTGGTGGGCTGCTGCTGCTATGGAA | –    | –    | –    | –    | –    | ++   | –    | ++   |
| Mut7| AGCTGGAGAGGGCTGCTGCTGCTATGGAA | –    | –    | –    | –    | –    | ++   | –    | ++   |
| Mut8| AGCTGGAGAGGGCTGCTGCTGCTATGGAA | –    | –    | –    | –    | –    | ++   | –    | ++   |

**B** EMSA with the same unlabeled probes

1  2  3  4  5  6  7  8

Fig. 5. Identification of the LRF-binding motif in the NRE of the COMP promoter. A. Sequences of the wild-type (Wt) and mutant (Mut) probes used in B. Mutant nucleotides are lowercase and underlined. The binding intensity of LRF to these probes is indicated: ++; stronger binding; +, binding; –, no binding. B. EMSA with the same protein fractions as in Fig. 3. Probes are as indicated above the lanes. The specific protein-DNA band is indicated by the arrow.

A consensus site might enhance the association of LRF with the consensus sequences.

LRF Inhibits COMP-specific Reporter Construct Activities and Endogenous COMP Gene Expression—To determine whether LRF represses transcription of the COMP promoter using reporter gene assays, four COMP-specific reporter gene plasmids (−1925COMPLuc, −1775COMPLuc, −Δ1925COMPLuc, and −592COMPLuc) were generated in which segments with or without an NRE from the 5′-flanking region of the COMP gene were linked to the upstream end of a region encoding luciferase in the pGL2-Basic vector, and one NRE-specific reporter construct (4xNRELuc) was generated in which a tandem repeat of four NREs was inserted upstream of the SV40 promoter in the pGL2-Promoter vector (Fig. 6A). We transfected RCS cells with these reporter constructs together with the mammalian expression plasmid pFLAG-LRF. As shown in Fig. 6B, LRF produced >90% inhibition of the reporter constructs with an NRE (−1925COMPLuc and −1775COMPLuc), and this repression was dose-dependent (Fig. 6D); as expected, LRF also significantly repressed the activity of 4xNREpGL2-Promoter (which contains four tandem repeats of NRE) (Fig. 6C), indicating that the NRE is sufficient for LRF binding in the transfected cells. Given that LRF also repressed the activity of two other reporter constructs without an NRE (−Δ1925COMPLuc and −592COMPLuc), but to a lesser degree (−40% inhibition) (Fig. 6B), we next examined whether LRF also binds to the proximal region of the COMP promoter using a magnetic bead assay. Both a 592-bp proximal region of the COMP promoter (−592 to −1) and a 400-bp distal region of the COMP promoter (−1925 to −1526) were labeled with biotin. Streptavidin-coupled magnetic beads conjugated to these biotin-labeled probes were incubated with nuclear extracts expressing pFLAG-LRF. Bound FLAG-LRF was detected by immunoblotting with anti-FLAG antibody. As shown in Fig. 6E, the NRE-containing distal region bound to LRF, but the proximal region did not, indicating that LRF cannot directly bind to the proximal region of the COMP promoter.

To confirm whether exogenous expression of LRF represses expression of the endogenous COMP gene, we transfected RCS cells, which express COMP, with the pFLAG-LRF expression plasmid and performed RT-PCR to determine the COMP mRNA levels. As shown in Fig. 6F, COMP gene expression was inhibited in the pFLAG-LRF-transfected RCS cells, in contrast with untransfected cells and cells transfected with the control pCMV vector.

**Generation of an LRF Stable Line in Which LRF Is Constitutively Overexpressed**—To determine whether stable overexpressed LRF affects the expression of endogenous COMP, we generated LRF stable lines in murine C3H10T1/2 progenitor cells, which undergo chondrogenic differentiation in the presence of inducers for chondrocyte differentiation. C3H10T1/2 cells were transfected with either an empty vector (for generating a control line) or the expression plasmid pFLAG-LRF together with the selective marker pMiniHgh, which confers hygromycin resistance upon transfected cells; the resultant clones were selected and amplified, and the level of ectopically expressed FLAG-LRF was examined by Western blotting with the anti-FLAG probe. As shown in Fig. 7, FLAG-LRF was clearly expressed in three selected LRF stable clones, i.e. clones 1, 9, and 11 (lanes 4–6, respectively), and absent in both parental C3H10T1/2 cells (lane 1) and the control stable lines pc1 and pc2 (lanes 2 and 3, respectively).

LRF Overexpression Inhibits Endogenous COMP Gene Expression—To determine the effects of stable overexpressed LRF on the expression of COMP, we first performed RT-PCR using micromass cultures of C3H10T1/2, control, and LRF stable lines exposed to recombinant BMP-2 for 1 week (Fig. 8A). COMP mRNA was expressed in the BMP-2-treated micromass cultures of parental C3H10T1/2 cells and the control stable lines pc1 and pc2 (lanes 1–3, respectively), whereas BMP-2-induced COMP expression in the course of chondrogenic differentiation was totally inhibited in LRF stable lines (lanes 4–6). We next performed Western blotting with anti-COMP antibodies using the same micromass cultures (Fig. 8B). In agreement with PCR data, the COMP protein was expressed in the control stable lines (lanes 1–3), but not in the LRF stable lines (lanes 4–6). Taken together, these results demonstrate that overexpression of LRF represses endogenous BMP-2-induced COMP expression.

LRF Overexpression Inhibits Chondrocyte Differentiation in C3H10T1/2 Micromass Cultures—In addition to COMP, several other extracellular matrix molecules of cartilage, such as aggrecan and collagen types II, IX, X, and XI, are hallmarks for monitoring the process of chondrogenic differentiation (60, 61). Type X collagen is indicative of hypertrophic chondrocytes (62, 63). We next performed RT-PCRs using the mRNAs isolated from micromass cultures of C3H10T1/2, control, and LRF stable lines exposed to recombinant BMP-2 for 1 week with the specific primers for collagen types II, IX, X, and XI; aggrecan; and GAPDH (used as an internal control). As shown in Fig. 9A, aggrecan and collagen types II, IX, X, and XI were expressed in the BMP-2-treated cultures (lanes 1–3), but expression of collagen type II, IX, and X mRNAs during BMP-2-induced chondrogenic differentiation was completely repressed in the presence of ectopically expressed FLAG-LRF (lanes 4–6). Although
aggrecan and type XI collagen mRNAs were still positive in the LRF stable lines (lanes 4–6), these levels were lower compared with the controls (lanes 1–3).

We also performed Alcian blue staining to confirm the repression of BMP-2-induced chondrogenesis by LRF overexpression using the micromass cultures of C3H10T1/2, control, and LRF stable lines exposed to recombinant BMP-2 for 2 weeks (Fig. 9B). Positive staining in the control lines (upper) was
COMP expression in C3H10T1/2 micromass cultures. Lysates prepared from the same cultures as peated studies. Control) was examined by RT-PCR. Similar results were obtained in repeated studies. mRNA expression of COMP and GAPDH (serving as an internal control) was examined by RT-PCR. Similar results were obtained in repeated studies. B, shown are the results from whole mount Alcian blue histochemistry. Staining was performed on the micromass cultures of control lines (C3H10T1/2, pc1, and pc2) or LRF stable lines (clones 1, 9, and 11) treated with 100 ng/ml recombinant BMP-2 for 14 days.

LRF Associates with HDAC1, and Its Repression Requires Deacetylase Activity—LRF contains a POZ domain, which is a conserved protein-protein interaction motif found in many transcription factors, and the most striking common property of the POZ domain-containing transcription factors is their ability to repress transcription via interaction with other key regulatory proteins such as HDACs (64, 65). A co-immunoprecipitation assay was first performed to determine whether LRF binds to HDAC1 in vitro (Fig. 10A). The cell extracts were first incubated with anti-LRF (lane 2) or anti-HDAC1 (lane 3) antibodies, and the immunoprecipitated complexes were detected with anti-HDAC1 antibody. A specific HDAC1 band was present in the immunoprecipitated complexes brought down by anti-LRF (lane 2) and anti-HDAC1 (lane 3) antibodies, but not by control IgG (lane 3), demonstrating that LRF associates with HDAC1 in vivo.

The interaction between LRF and HDAC1 was confirmed using an in vitro protein-protein interaction assay (an in vitro GST pull-down assay) (Fig. 10B). Briefly, affinity-purified GST as well as N-terminally (aa 51–482) and C-terminally (aa 1–432) truncated HDAC1 fused to GST that was immobilized on glutathione-Sepharose beads were incubated with in vitro translated LRF and, after washing, resolved by Western blotting. Similar to the GST control (lane 2), C-terminally truncated GST-HDAC1-(1–432) did not pull down the LRF protein (lane 4), whereas N-terminally truncated GST-HDAC1-(51–482) efficiently pulled down LRF (lane 3), indicating that the C-terminal (but not N-terminal) 50 amino acids of HDAC1 contain LRF-binding domains. To narrow down the LRF-binding sequences in the C terminus of HDAC1, two additional mutants were generated, and the same in vitro binding assay was performed (Fig. 10C). Removal of 15 amino acids from the C terminus (compare GST-HDAC1-(51–467) with GST-HDAC1-(51–482)) did not affect interaction, nor did further removal of the other 15 amino acids from the C terminus (GST-HDAC1-(1–432)). Given that mutant HDAC1-(1–432) failed to bind LRF (Fig. 10B, lane 4), it appears that 20 amino acids (aa 439–452) of HDAC1 are essential for interacting with LRF.

To determine whether the LRF-HDAC1 protein complex is detectable in the NRE of the COMP promoter, ChIP assays were performed in C3H10T1/2 progenitor cells, which express both LRF and HDAC1. Endogenous HDAC1-LRF-DNA complexes were immunoprecipitated with control IgG (negative control) (Fig. 10D, lane 3), anti-LRF antibody (positive control) (lane 2), or anti-HDAC1 antibody (lane 4), and the DNA purified from these coprecipitations and input DNA (lane 1) were analyzed by PCR. We observed amplifications of COMP promoter DNA from input DNA and DNAs isolated from both
anti-LRF and anti-HDAC1 antibody-precipitated (but not control IgG-precipitated) complexes. These results demonstrate that the LRF-HDAC1 complex is detectable in the NRE of the COMP gene promoter.

To examine whether HDACs are required for the LRF-mediated repression of the COMP reporter construct, RCS cells transfected with the COMP-specific reporter construct (1925COMP luc) and the mammalian expression plasmid pFLAG-LRF were cultured in the presence of different concentrations of TSA for 48 h, and luciferase and β-galactosidase activities were measured. As shown in Fig. 10E, LRF inhibition of the COMP promoter was attenuated when TSA was added to the medium, demonstrating that HDACs are involved in the LRF-mediated repression of COMP promoter activity.

We next tested whether COMP gene expression in C3H10T1/2 cells can be stimulated by TSA. As revealed by RT-PCR (Fig. 10F), COMP mRNA was detectable in the TSA-treated (but not untreated) C3H10T1/2 cells, indicating that TSA induces COMP gene expression and that this induction is dose-dependent.

DISCUSSION

Our previous studies identified an NRE located between −1775 and −1746 of the 5′-flanking region of the murine COMP gene (35, 39). In this study, a yeast one-hybrid screen was used to identify transcription factors that bind to this NRE. We present comprehensive evidence that LRF, which contains a POZ domain, a conserved 120-amino acid motif involved in transcriptional repression and dimerization, and four Krüppel-like zinc finger DNA-binding motifs (44), binds directly to the NRE in the promoter of the COMP gene. We also present evidence that repression of the COMP promoter by LRF blocks both endogenous COMP gene expression as well as chondrogenesis of C3H10T1/2 micromass cultures. The molecular mechanism underlying LRF-mediated gene transcription appears to involve the association of LRF with HDAC1.

Although a 9-bp guanidine-rich LBE (GAGGGTCCC) in the 30-bp NRE (identified via an EMSA with a series of mutant NRE probes) of the mouse COMP promoter is essential for binding to LRF, the following six nucleotides (GGTCCC) also appear to be involved in the association between LRF and the
LRF, binds to both the cKrox tandem site and the Egr-1 single flexible manner (58, 59) and that OCZF, the rat homolog of LRF, does in mouse. It has also been reported that FBI-1 binds to DNA in a flexible manner (58, 59) and that OCZF, the rat homolog of LRF, is highly expressed in osteoclasts, and antisense OCZF cDNA suppresses the formation of osteoclast-like multinucleated cells in bone marrow culture (48). These results suggest that OCZF plays an important role in the late stage of osteoclastogenes. LRF was reported to repress the promoter activity of several extracellular genes (66). Our unbiased genetic screen and EMSA as well as ChIP showed that LRF directly binds to the NRE in the promoter region of the COMP gene and associates with HDAC1, and our functional assays demonstrated that LRF regulates COMP gene expression and chondrogenesis of high density micromass cultures of C3H10T1/2 cells.

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REFERENCES

1. Ferguson, C. M., Miclau, T., Ha, D., Alpern, E., and Helms, J. A. (1998) Annu. N. Y. Acad. Sci. 857, 33–42
2. Erlebacher, A., Filvaroff, E. H., Gitelman, S. E., and Derynck, R. (1995) Cell 80, 371–378
3. Di Cesare, P. E., Morgelin, M., Mann, K., and Paulsson, M. (1994) Eur. J. Biochem. 223, 927–937
4. Di Cesare, P., Hauser, N., Lehman, D., Pasumarti, S., and Paulsson, M. (1994) FEBS Lett. 354, 237–240
5. Di Cesare, P. E., Carlson, C. S., Stollerman, E. S., Chen, F. S., Leslie, M., and Perras, R. (1997) FEBS Lett. 412, 249–252
6. Di Cesare, P. E., Fang, C., Leslie, M. P., Tulli, H., Perras, R., and Carlson, C. S. (2000) J. Orthop. Res. 18, 713–720
7. Di Cesare, P. E., Fang, C., Leslie, M. P., Della Valle, C. J., Gold, J. M., Tulli, H., Perras, R., and Carlson, C. S. (1999) J. Orthop. Res. 17, 437–445
8. Di Cesare, P. E., Carlson, C. S., Stollerman, E. S., Hauser, N., Tulli, H., and Paulsson, M. (1996) J. Orthop. Res. 14, 946–955
9. Hedbom, E., Antonsson, P., Hjerpe, A., Aeschliann, D., Paulsson, M., Rosa-Pimentel, E., Sommarin, Y., Wendel, M., Oldberg, A., and Heinegard, D. (1996) J. Biol. Chem. 271, 6125–6136
10. Morgelin, M., Engel, J., Heinegard, D., and Paulsson, M. (1992) J. Biol. Chem. 267, 14275–14284
11. Shen, Z., Heinegard, D., and Sommarin, Y. (1995) Matrix Biol. 14, 733–738
12. Efimov, V. P., Engel, J., and Malashkevich, V. N. (1996) Proteins 24, 259–262
13. Kajava, A. V. (1996) Proteins 24, 218–226
14. Di Cesare, P. E., Morgelin, M., Carlson, C. S., Pasumarti, S., and Paulsson, M. (1995) J. Orthop. Res. 13, 422–428
15. Kawai, H., Nichimura, G., Watanabe, S., Mabuchi, A., Ikeda, T., Ohashi, H., Sasaki, A., Tano, T., and Ikegawa, S. (2002) Skelet. Radiol. 31, 739–737
16. Hashimoto, Y., Tomiyama, T., Yamano, Y., and Mori, H. (2003) Am. J. Pathol. 163, 101–110
17. Nakayama, H., Endo, Y., Aota, S., Sato, M., Fujita, T., and Kikuchi, S. (2003) Oncol. Rep. 10, 871–875
18. Song, H. R., Lee, K. S., Li, Q. W., Koo, S. K., and Jung, S. C. (2003) J. Hem. Genom. 48, 222–225
19. Mabuchi, A., Manabe, N., Haga, N., Kitis, H., Ikeda, T., Kawai, H., Tamai, K., Hamada, J., Nakamura, S., Brunetti-Pierri, N., Kinimaki, M., Takatori, Y., Nakamura, K., Nichimura, G., Ohashi, H., and Ikegawa, S. (2003) Hum. Genet. 112, 84–90
20. Kleerebeumper, Q., Hecht, J. T., and Patkey, J. A. (2002) J. Biol. Chem. 277, 10581–10589
21. Briggs, M. D., Bursmussen, I. M., Weber, J. L., Yuen, J., Reinker, K., Garber, A. P., Rimoin, D. L., and Cohn, D. H. (1993) Genomics 18, 656–660
22. Briggs, M. D., Hoffman, S. M., King, L. M., Olsen, A. S., Mohrenweiser, H., Leroy, J. G., Mortier, G. R., Rimoin, D. L., Lachman, R. S., and Gaines, E. S. (1995) Nature 369, 330–336
23. Briggs, M. D., Mortier, G. R., Cole, W. G., King, L. M., Gokh, S. S., Bonaventure, J., Nuytink, L., De Paepe, A., Leroy, J. G., Biesecker, L., Lipson, M., Wilcox, W. R., Lachman, R. S., Rimoin, D. L., Knowlton, R. G., and Cohn, D. H. (1998) Am. J. Hum. Genet. 62, 311–319
24. Hecht, J. T., Francomano, C. A., Briggs, M. D., Deere, M., Conner, B., Horton, W. A., Warman, M., Cohn, D. H., and Blanton, S. H. (1993) Genomics 18, 661–666
25. Hecht, J. T., Nelson, L. D., Crowder, E., Wang, Y., Elder, P. F., Harrison, W. R.,
Leukemia/Lymphoma-related Factor, a POZ Domain-containing Transcriptional Repressor, Interacts with Histone Deacetylase-1 and Inhibits Cartilage Oligomeric Matrix Protein Gene Expression and Chondrogenesis

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