Insulation of the Ubiquitous Rxrb Promoter from the Cartilage-specific Adjacent Gene, Col11a2*§

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The retinoid X receptor β gene (Rxrb) is located just upstream of the α2(XI) collagen chain gene (Col11a2) in a head-to-tail manner. However, the domain structures of these genes are unknown. Col11a2 is specifically expressed in cartilage. In the present study, we found Rxrb expression in various tissues with low expression in the cartilage. Col11a2 1st intron enhancer directed cartilage specific expression when linked to the heterologous promoter in transgenic mice. These results suggest the presence of enhancer-blocking elements that insulate Rxrb promoter from the Col11a2 enhancer. So far, most of insulators examined in vertebrates contain a binding site for CTCF. We found two possible CTCF-binding sites: one (11P) in the intergenic region between Rxrb and Col11a2 by electrophoretic mobility shift assays, and the other in the 4th intron of RXRB by data base search. To examine the function of these elements, we prepared bacterial artificial chromosome (BAC) transgene constructs containing a 142-kb genomic DNA insert with RXRB and COL11A2 sequences in the middle. Mutation of 11P significantly decreased the RXRB promoter activity in muscular cells and significantly increased expression levels of RXRB in chondrosarcoma cells. In transgenic mouse assays, the wild-type BAC transgene partly recapitulated endogenous Rxrb expression patterns. A 507-bp deletion mutation including 11P enhanced the cartilage-specific activity of the RXRB promoter in BAC transgenic mice. Chromatin immunoprecipitation analysis showed that CTCF was associated with RX4, but not with 11P. Our results showed that the intergenic sequence including 11P insulates Rxrb promoter from Col11a2 enhancer, possibly associating with unknown factors that recognize a motif similar to CTCF.

Cartilage is a highly specialized tissue that serves as the template for skeletal development and lines the joint surface. Cartilage consists of an abundant extracellular matrix maintained by chondrocytes. The collagen network provides scaffolding for proteoglycans in the extracellular matrix and confers tensile strength important for resisting compression and shearing loads in cartilage. Cartilage collagen fibrils are heterotypic fibrils composed of types II, IX, and XI collagens. The type XI collagen molecules co-assemble stoichiometrically with the major collagen, type II collagen, to form cartilage fibrils, whereas type IX collagen is associated with the surface of the fibrils (1, 2). The type XI collagen molecule is composed of three distinct subunits: α1(XI), α2(XI), and α3(XI) (3). Type XI collagen likely regulates the diameter of cartilage collagen fibrils (4). Expression of the α2(XI) collagen chain gene is highly specific to cartilage (5). Mutations in the α2(XI) collagen chain gene are associated with certain forms of human chondrodysplasia, Stickler syndrome and otospondylomegalysepsydysplasia, indicating that type XI collagen is intimately involved in skeletal morphogenesis (6).

We previously cloned the mouse α2(XI) collagen chain gene (Col11a2) (7). We found that when the 1st intron sequence of Col11a2 was linked to the Col11a2 autologous basal promoter, it directed cartilage-specific expression in transgenic mice, indicating that the 1st intron sequence is a cartilage-specific enhancer (8). We also found that the retinoid X receptor β gene (Rxrb) is located just upstream of Col11a2 in a head-to-tail manner. This alignment is conserved in organisms ranging from humans to zebrafish. The distance between the termination codon of Rxrb and the ATG codon of Col11a2 is 2,441 bp in humans, 2,161 bp in rats, 2,123 bp in mice, and 13,800 bp in zebrafish. The COL11A2 is 30-kb in size, consisting of more than 50 exons and RXRB is 7-kb in size, consisting of 10 exons. The three retinoid X receptors, RXRα, RXRβ, and RXRγ, belong to the family of nuclear hormone receptors that are ligand-activated transcription factors (9). RXR is an obligatory component of various nuclear receptor heterodimers such as the retinoic acid receptor, vitamin D receptor, thyroid hormone receptor, and peroxisome proliferator activated receptor RXRs play an integrative role in a number of nuclear receptor-mediated pathways. RXRα and RXRβ transcripts are widely expressed in embryo and adult tissues, whereas the distribution of RXRγ transcripts is more restricted (10). Approximately 50% of mice lacking RXRβ died before birth or at birth. Male mice

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4 and Tables S1–S4.

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‡‡ The abbreviations used are: Rxrb, retinoid X receptor β gene; BAC, bacterial artificial chromosome; BORIS, Brother of the Regulator of Imprinted Sites; Chip, chromatin immunoprecipitation; Col11a2, α2(XI) collagen chain gene; CTCF, CCCTC-binding factor; EMSA, electrophoretic mobility shift assay; RCS, rat chondrosarcoma; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; NF, neurofilament; RXR, retinoid X receptor; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AD, adenovirus.
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that survived were sterile due to oligoasthenoteratozoospermia (11). RXRβ and RXRγ are expressed in the striatum and control the function of the dopaminergic mesolimbic pathway (12). RXR forms heterodimers with liver X receptor and farnesoid X-activated receptor and regulates cholesterol balance (13). Thus, the fidelity of the spatiotemporal expression of RXRβ is crucial for the normal development of a wide variety of tissues.

The organization of eukaryotic genomes necessarily results in the proximity of domains with distinct functions. The identity of domains is maintained by classical transcriptional regulatory elements, such as enhancers, silencers, and upstream activating sequences. In some cases, specific DNA sequences, referred to as insulators (14), and their associated binding proteins have a role in establishing or maintaining discrete inter-domain boundaries. Insulators have been divided into two classes: enhancer-blocking insulators, which prevent distal enhancers from activating a promoter when placed between an enhancer and promoter, and barrier insulators, which block heterochromatinization and the consequent silencing of a gene (15). In vertebrates, the enhancer blocking activity of insulators is associated with a binding site for the CCCTC-binding factor (CTCF), which recognizes long and diverse nucleotide sequences. CTCF is a ubiquitously expressed nuclear protein with 11 zinc finger DNA-binding domains. Thus far, CTCF remains as the only major protein implicated in the establishment of insulators in vertebrates (16). There has been great interest in identifying other binding sites for CTCF. Several recent high-throughput ChIP-chip analyses and comparative genomic studies have identified tens of thousands of potential CTCF-binding sites in the human and mouse genomes (17–19). The sensitivity of the ChIP-chip analysis for CTCF is 88%; thus, there are some false-negative results (18). Recently, it was discovered that cohesin binds to many of the CTCF-binding sites, co-localizing with CTCF to insulate promoters from distinct enhancers (20, 21).

Despite the proximity of Rxrb and Col11a2, the dissimilarities in their expression patterns and functions suggest the existence of an intergenic boundary. To investigate this possibility, we first clarified differences in the expression patterns of the two genes and examined whether the cartilage-specific enhancer of Col11a2 could affect transcriptional activities of heterologous promoters. We then searched for a CTCF-binding site between the two genes. We examined whether the intergenic sequence and CTCF-binding site affected the activities of the Rxrb promoter by using bacterial artificial chromosome (BAC) transgene constructs that cover the entire Rxrb and Col11a2 genes.

EXPERIMENTAL PROCEDURES

Northern Hybridization—Total RNA was extracted from various mouse tissues at 16.5 days postcoitus and from cell lines by using RNeasy Mini Kits (Qiagen, Santa Clara, CA). Ten micrograms of total RNA was fractionated by electrophoresis through formaldehyde-gelose gels and then transferred onto Nytran membranes (Amersham Biosciences). Complementary DNAs (cDNAs) were labeled with [35S]dCTP by using the Rediprime II Random Prime Labeling System (Amersham Biosciences). The membranes were hybridized with 32P-labeled mouse Col11a2 and Rxrb cDNAs.

Real-time RT-PCR—Total RNAs were digested with DNase to eliminate any contaminating genomic DNA before real-time quantitative RT-PCR. One microgram of total RNA was reverse transcribed into first-strand cDNA by using QuantiTect Reverse Transcription (Qiagen). The PCR amplification proceeded in a 20-μl reaction mixture containing 2 μl of cDNA, 10 μl of SYBR PremixExTaq (Takara, Japan), and 4 pmol of primers specific for rat Rxrb and Col11a2. The quantified individual RNA expression levels of rat Col11a2 and rat Rxrb were normalized to the respective rat Gapdh expression levels. The primers used are listed in supplemental Table S1.

Cell Lines and Cell Culture—RCS (rat chondrosarcoma) cells (22), L6 (rat skeletal muscle) cells (23), and FR (rat skin fibroblast) cells (ATCC number CRL-1213) (24) were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin. H411E (rat hepatoma) cells (25) were cultured in α-minimal essential medium with 10% fetal bovine serum and 1% streptomycin/penicillin. PC12 (rat adrenal pheochromocytoma) cells (ATCC number CRL-1721) (26) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 10% horse serum, and 1% streptomycin/penicillin in collagen dishes. L6, FR, and H411E cells were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan).

Histology and in Situ Hybridization—Mouse embryos were dissected with a stereomicroscope, fixed in 4% paraformaldehyde, processed, and embedded in paraffin. Serial sections were stained with hematoxylin and eosin. RNA in situ hybridization was performed by using 35S-labeled antisense riboprobes as previously described (40). Probes included mouse Col11a2 cDNA (pRAC2-28) (7) and Rxrb cDNA (residues 910–1341, GenBank™ accession number M84818).

Generation of Transgenic Mice Bearing the Col11a2 Enhancer Linked to Heterologous Promoters—pAD-LacZ was the expression vector pADbeta (Clontech number 6176-1), which contains the adenovirus II major promoter, SV40 RNA splice site, the β-galactosidase reporter gene and the SV40 polyadenylation signal. The fragment of 2.3 kb of the first intron sequence of Col11a2 as an enhancer was cloned into the Sall/PstI poly linker site located downstream of the SV-40 polyadenylation signal of Ad-beta to create pAD-LacZ-Int. Nf-LacZ-Int was created by inserting the 2.3-kb fragment of the first intron sequence of Col11a2 as an enhancer into the Sall/PstI poly linker site located downstream of the SV-40 polyadenylation signal of pNf-LacZ (27), which contains the neurofilament promoter, SV40 RNA splice site, the β-galactosidase reporter gene, and the SV40 polyadenylation signal.

The plasmids AD-LacZ, AD-LacZ-Int, and Nf-LacZ-Int were digested with EcoRI and PstI to release the inserts from their vector sequences. Transgenic mice were produced by microinjecting each of the inserts into the pronuclei of fertilized eggs from F1 hybrid mice (C57BL/6x DBA) as described previously (8). Generation 0 (G0) embryos were sacrificed at 13.5 days postcoitus and processed for expression analysis of the reporter gene. Transgenic embryos were identified by PCR analysis of genomic DNA extracted from the placenta or tail as described.
previously (8). X-Gal staining of mouse bodies and sections was performed as previously described (28).

Electrophoretic Mobility Shift Assays—The full-length coding region of mouse CTCF cDNA was PCR amplified with primers NT364 and NT365 (supplemental Table S2) and cloned into pCR-BluntII-TOPO using the Zero Blunt TOPO PCR cloning kit (Invitrogen catalog number K2800-20). CTCF cDNA was transferred to pTNT vector and used for in vitro synthesis of CTCF protein with the TnT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, catalog number L1170). Nuclear extracts from RCS cells were prepared using Celllytic NuCLEAR Extraction kits (Sigma) according to the manufacturer’s instructions. Probes were prepared as described under supplementary data.

DNA fragments were denatured, end-labeled with [γ-32P]ATP and T4 polynucleotide kinase, and annealed to prepare radiolabeled probes. The radiolabeled probes were gel purified and combined with equal amounts of in vitro-synthesized CTCF protein or equal amounts of nuclear extracts. For binding reactions, we used a buffer containing phosphate-buffered saline with 5 mM MgCl₂, 0.1 mM ZnSO₄, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 10% glycerol in the presence of 10 ng/μl poly(dI-dC) and unlabeled 10 ng/ml double-strand synthesized oligonucleotides containing an Sp1-binding site, as Sp1-like factors can bind to GC-rich segments and obscure the binding of CTCF (29). The reaction mixtures of a 10-μl final volume were incubated for 20 min at room temperature followed by electrophoresis on 6% non-denaturing polyacrylamide gels. We performed electrophoretic mobility shift assay (EMSA) with nuclear extracts in the presence of 0.2 ng/μl salmon sperm DNA. For super-shift assays, the reaction mixture was combined with 2 μl of anti-CTCF antibody (Upstate number 07-729) or normal rabbit IgG and incubated for 2 h at 4°C before the addition of radiolabeled probes. The primers and synthesized oligonucleotides are listed in supplemental Table S2.

ChIP Assay—We used the ChIP Assay Kit (Upstate number 17-295), according to the manufacturer’s protocol as described under supplementary data.

We performed quantitative real-time PCR in triplicate with DNA prepared with anti-acetylated histone H3 antibody and input DNA. We used the LightCycler FastStart DNA MasterPlus HybProbe kit (Roche number 03-515-575001) in a LightCycler (Roche). Amplification reactions were performed in a volume of 20 μl with 20 pm (each) primers and FRET probes (0.4 μl of each primer per reaction), 5 μl of template DNA, and 1 × Master mixture (4 μl/reaction).

We performed quantitative real-time PCR in triplicate with DNA prepared with anti-CTCF antibody, DNA prepared with anti-Rad21 antibody, DNA prepared with anti-BORIS antibody, and input DNA by using primer pairs designed for 11P and RX4. We used LightCycler and SYBR Green reagent (Takara), according to the manufacturer’s instructions.

Data were analyzed with LightCycler software by the Fit Point method to minimize noise and obtain the best possible correlation coefficient between standards. The -fold difference for a particular target sequence was determined by calculating the ratio of the amount of the target sequence in the immuno-precipitation to the amount of the target sequence in the input DNA. Primers and FRET probes were selected from human and rat retinoid X receptor and α2(XI) collagen chain gene sequences using Roche Primer design software. Primers and FRET probes were obtained from Sigma and Nihon Gene Research Laboratories Inc. (Sendai, Japan), respectively. The primers and FRET probes are listed in supplemental Table S3.

BAC Transgene Construction—A human BAC clone, CTDA-2054115 (WT), which contains RXRB and COL11A2 in the middle, was purchased from Invitrogen. Modifications of a BAC clone were performed as described under supplementary data. Constructs were checked with digestion with various restriction enzymes and PCR analysis. The primers used in BAC modifications are listed in supplemental Table S4.

Colonies—Two micrograms of the BAC transgene constructs (WT-Pur, 11Psub-Pur, 11Pdel-Pur, and RX4sub-Pur) were transfected into 1 × 10⁶ L6 cells using Lipofectamine 2000 (Invitrogen) in 10-cm dishes (supplemental Fig. S2). After 24 h, cells were selected by puromycin (5 μg/ml). After 10 days of selection, dishes were stained with crystal violet and the colonies were counted.

Establishment of Transgenic RCS Cells—The BAC transgene constructs (SV40-Pur/WT, SV40-Pur/11Psub, and SV40-Pur/11Pdel) were transfected into 1 × 10⁶ RCS cells in 10-cm dishes with Lipofectamine 2000 (Invitrogen) (supplemental Fig. S2). After 24 h, cells were selected by puromycin (3 μg/ml). After 14 days of selection, colonies were picked-up and replated in 24-well dishes. We established 12, 14, and 18 independent RCS cell lines for SV40-Pur/WT, SV40-Pur/11Psub, and SV40-Pur/11Pdel transgenes, respectively. Total RNAs were extracted from each transgenic cell and subjected to real-time RT-PCR to analyze the relative expression levels of rat Col11a2, rat Rxrb, human COL11A2, and human RXRB mRNAs. We normalized the quantified individual RNA expression levels of rat Col11a2 and rat Rxrb to the respective rat Gapdh expression levels, and those of human COL11A2 and human RXRB were normalized to the respective puromycin expression levels. The primers used are listed in supplemental Table S1.

Production and Genotyping of Transgenic Mouse Lines—BAC transgene constructs (WT-LacZ, 11Psub-LacZ, 11Pdel-LacZ, and RX4sub-LacZ) were purified with the Qiagen large clone kit were performed as described under supplementary data. Colony colonies were performed as described under supplementary data.

RESULTS

Different Expression Patterns between the Rxrb and the Col11a2 Genes—To delineate differences in the transcriptional activities of Rxrb and Col11a2 in various tissues, we analyzed the expression of mRNAs of these genes in samples from identical tissues or cells. Northern hybridization analysis on tissue
samples showed that Col11a2 mRNAs were abundant in limb buds but not in the intestine, brain, liver, or lung of mouse embryos at 16.5 days postcoitus (Fig. 1A). The Rxb mRNAs were clearly detected in all of these tissues. Northern hybridization analysis on cultured cells showed that Col11a2 mRNA was abundant in RCS but not in L6 (rat skeletal muscle), FR (rat fibroblast), PC12 (rat adrenal pheochromocytoma), and H4IIE (rat hepatoma) cells. The Rxb mRNAs were clearly detected in all these cells except for the RCS cells. Real-time RT-PCR analysis on cultured cells revealed that relative mRNA expression levels of Col11a2 to Gapdh were much higher in RCS as compared with the levels in L6, FR, PC12, and H4IIE cells (Fig. 1B). The relative mRNA expression levels of Rxb were low in RCS cells as compared with the levels in other cells. In situ hybridization performed on semi-serial sections showed that Col11a2 mRNA was highly and specifically expressed in cartilage (Fig. 1C). Rxb mRNA was detected in various tissues, with abundant expression in muscle. Signals for Rxb mRNA were present in cartilage; these signals were weak compared with the signals in other tissues. In summary, we confirmed the previous reports that the Col11a2 gene was transcribed specifically in cartilage. We found that Rxb was expressed in a variety of tissues; interestingly, Rxb was weakly expressed in cartilage.

Lysine residues on the N-terminal tails of histones H3 and H4 are more highly acetylated in the neighborhood of transcriptionally active promoters and enhancers than those in regions of transcriptional inactivity (30). We examined the acetylation levels and patterns of histone H3 from the Rxb to Col11a2 genes using RCS and L6 cells (Fig. 2A). A ChIP assay with anti-acetylated histone H3 antibody showed that the promoter region of Rxb and the promoter and first intron regions of Col11a2 were hyperacetylated as compared with other regions in RCS cells. Acetylation levels of histone H3 were low except for the promoter region of Rxb in L6 cells; these results are consistent with the expression levels of Rxb and Col11a2 in L6 cells.

Effects of the First Intron Sequence of Col11a2 on Transcriptional Activities of Heterologous Promoters—

Some enhancers are specific for certain promoters (31, 32). We examined whether the 1st intron sequence of Col11a2 serves as an enhancer for heterologous promoters by generating transgenic mice bearing LacZ reporter gene constructs. The adenovirus late II major promoter (AD) directed LacZ expression without tissue specificity. We obtained 16 founder mice bearing an AD-LacZ transgene. Eight of the mice showed X-gal staining, and the patterns of X-gal staining varied among the founder mice (Fig. 2B), suggesting the influence of integration sites of the transgene in the genome. Addition of the 1st intron sequence of Col11a2 to the AD promoter resulted in the production of transgenic mice with cartilage-specific expression of LacZ (Fig. 2C).
obtained 8 founder mice bearing an AD-LacZ-Int transgene. Four of these mice showed cartilage-specific LacZ expression, 2 showed LacZ expression in non-cartilaginous tissues, and 2 showed no X-gal staining. Neurofilament gene promoter (Nf) directs neural tissue-specific expression of the LacZ reporter gene in transgenic mice (33). The addition of the 1st intron sequence of Col11a2 to the neurofilament promoter resulted in the production of transgenic mice with cartilage-specific expression of LacZ (Fig. 2D). We obtained 7 founder mice bearing the Nf-LacZ-Int transgene. Three of these mice showed cartilage-specific LacZ expression, 1 showed LacZ expression in various tissues including cartilage and brain, and three showed no X-gal staining. Although many of mice bearing transgenes with the Col11a2 1st intron sequence directed expression primarily in cartilage, some mice showed expression in non-cartilaginous tissues. Because expression of the transgene is affected by the site where the transgene is integrated in the genome, it is possible to speculate that the sequences around the integrated sites might have enhancer activities in non-cartilaginous tissues. Overall, the results suggest that the 1st intron of Col11a2 could serve on heterologous promoters as an enhancer in cartilage, because AD-LacZ and NF-LacZ transgenic mice do not show the cartilage-specific expression pattern of LacZ. These results raise the possibility of the existence of a mechanism that prevents the enhancer of the 1st intron sequence of Col11a2 from affecting the Rxrb promoter in the genome.

CTCF-binding Sites in the Intergenic Region between Rxrb and Col11a2—We performed EMSAs to identify CTCF binding sites in the intergenic region between the rat Col11a2 and Rxrb genes. We first prepared 12 radiolabeled, overlapping probes that cover the stretch from the termination codon of Rxrb to the translational start site (ATG codon) of Col11a2 (Fig. 3A). We incubated the probes with in vitro translated CTCF protein. We detected the shifted bands reproducibly only in samples containing probe 8 and DNA fragments with the CTCF-binding
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We prepared human BAC clone CTD-2054I15 (Invitrogen) that contained a 142-kb genomic DNA insert with RXRB and COL11A2 sequences in the middle. By using BAC recombineering techniques, we introduced a 14-bp substitution mutation at 11P, a 507-bp deletion mutation including the 11P site, and a 14-bp substitution mutation at the RX4 site to prepare 11Psib, 11Pdel, and RX4sib constructs, respectively (Fig. 4A). The sequences of the 14-bp substitution mutations in 11Psib and RX4sib were identical to those of probes h-M5Bgl and 13M, respectively, which we used in EMSA and thus contained BglII cleavage sites. The 507-bp deleted sequence in 11Pdel corresponded to -1148 to -642 of the translational start site of human COL11A2. Previous reports showed tissue-specific regulatory elements (27) and a Sox9-binding element (34) in the intergenic sequence between mouse Rxrb and Col11a2. The human sequences corresponding to these elements reside at -1148 to -1125 and -1123 to -1107 from the translational start site, respectively. Thus, these elements in addition to 11P were deleted in 11Pdel. Next, we inserted a puromycin-resistance gene sequence into the 1st exon of RXRB in the original BAC clone (WT), 11Psib, 11Pdel, and RX4sib

FIGURE 3. EMSA. A, a region from the last exon of Rxrb to the first exon of Col11a2. The positions of the DNA fragments used for EMSA are indicated as 1–12 and A–D. B, screening for CTCF-binding sites by EMSA with probes 1–12 and in vitro translated CTCF. C, the specific interaction of probe B with in vitro translated CTCF. D, the coding strand sequence of the rat 8 probes, rat mutated probes (M1–8 and M5Bgl), human h-B probes and human mutated probes (h-M5Bgl). Only mutated nucleotides are shown. BglII sites were underlined. E, probe M5 did not interact with CTCF. Probe M4 interacted with CTCF to a lesser extent than the other mutated probes. F, an excess of unlabeled probes M4 and M5 failed to block the binding of in vitro translated CTCF to probe B, whereas other probes abolished the binding. G, rat probe B and human probe h-B specifically interacted with endogenous CTCF in nuclear extracts of RCS cells. H, positions of possible CTCF-binding sites. Half-arrows indicate CTCF-binding sites by data base search. The open arrow indicates the CTCF-binding site found by EMSA in this study. i, the specific interaction of probe 13 with in vitro translated CTCF. P, DNA fragments with the CTCF-binding site DMD4 at the mouse Col11a2 promoter, arrows, shifted CTCF-DNA complexes; FII, DNA fragments containing CTCF-binding site FII at the chicken alpha-globin locus; a-CTCF, anti-CTCF antibodies; control, in vitro translation reaction mixture with empty vector; r-CTCF, in vitro translated CTCF; asterisk, super-shifted complexes; arrowheads, shifted nuclear extract-DNA complexes; vertical half-arrows, CTCF-binding sites by data base; open arrow, CTCF-binding site by EMSA (11P).

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FIGURE 4. 11P affected the Rxrb promoter activities in L6 cells and RCS cells. A, schematic representation of mutations introduced into the BAC clone, CTD-2054I15. We introduced a 14-bp substitution mutation at 11P, a 507-bp deletion mutation including the 11P site, and a 14-bp substitution mutation at the RX4 site to prepare 11Psub, 11Pdel, and RX4sub constructs, respectively. Mutated sequences in 11Ppub and RX4sub contained BglII cleavage sites. B and C, colony assays in L6 cells. B, to monitor transcriptional activities of the RXRB gene in colony assays, we inserted the puromycin-resistance gene sequence into the 1st exon of RXRB in each construct to generate WT-Pur, 11Psub-Pur, 11Pdel-Pur, and RX4sub-Pur transgenes, respectively. C, 2 μg of each transgene was introduced into L6 cells. Cells were then cultured in the presence of puromycin and stained with crystal violet, and the numbers of colonies were counted. Error bars indicate the mean ± S.D. (n = 3), *p < 0.05 and **p < 0.01 as determined by the Student’s t test. D, establishment of transgenic RCS cells. D, we inserted a SV40 early promoter–puromycin resistance gene cassette into each construct to generate SV40-Pur/WT, SV40-Pur/11Psub, and SV40-Pur/11Pdel (Fig. 4D). We introduced these constructs into RCS cells that were cultured in the presence of puromycin until colonies formed. Isolated colonies were picked up and transferred to separate dishes to establish stably transformed RCS cells bearing the BAC transgene constructs. We extracted total RNAs from each transfectant and performed real-time RT-PCR. PCR with human primers showed that there were no significant differences in the mean relative expression levels of human COL11A2 mRNAs to puromycin mRNAs between RCS cells bearing SV40-Pur/WT, SV40-Pur/11Psub, and SV40-Pur/11Pdel and RCS cells bearing SV40-Pur/WT. The mean relative expression levels of human RXRB mRNAs to puromycin mRNAs were significantly higher in RCS cells bearing SV40-Pur/11Psub or SV40-Pur/11Pdel than in RCS cells bearing SV40-Pur/WT (Fig. 4E). There was no amplification with human primers and cDNAs from the original RCS cells as templates, suggesting the specific amplification of human transgene products (Fig. 4F). PCR with rat primers demonstrated that there were not significant differences in the mean relative expression levels of rat endogenous Col11a2 or rat endogenous Rxrb mRNAs to rat Gapdh mRNAs between RCS cells bearing SV40-Pur/11Psub or SV40-Pur/11Pdel and RCSs bearing SV40-Pur/WT (data not shown). These results suggest that 11P is a silencer for the RXRB promoter in RCS cells. Alternatively, 11P might block the enhancer activity of the Col11a2 gene from affecting the Rxrb promoter in RCS cells.

Effect of CTCF-binding Sites and the Intergenic Sequence on RXRB Promoter Activity in Transgenic Mice—To examine the effects of the intergenic sequence between RXRB and COL11A2 and CTCF-binding sites on the RXRB promoter activities in vivo, we generated transgenic mice bearing BAC DNA containing RXRB and COL11A2 genes. We inserted a LacZ sequence into the 1st exon of RXRB in each of the WT, 11Psub, 11Pdel, and RX4sub constructs to generate WT-LacZ, 11Psub-LacZ, 11Pdel-LacZ, and RX4sub-LacZ transgenes, respectively (Fig. 5A). LacZ was used to monitor RXRB promoter activity. The

were checked by electrophoresis after digestion with BglII (supplemental Fig. S1). Lipofectamine 2000 was used to introduce 2 μg of each transgene into L6 cells, and the cells were cultured in the presence of 5 μg/ml of puromycin for 7 days. The mean numbers of colonies formed in cultures transfected with 11Psub-Pur or 11Pdel-Pur were significantly decreased compared with the colonies in cultures with WT-Pur (Fig. 4C). The mean number of colonies formed in cultures transfected with RX4-Pur was not significantly different from the number in WT-Pur cultures. These results suggest that 11P is an enhancer for the RXRB promoter in L6 cells. Alternatively, 11P might block the silencer activity of the Col11a2 gene from affecting the Rxrb promoter in L6 muscular cells.

Mutation of 11P Increased RXRB Expression in Transgenic RCS Cells—We also performed colony assays on RCS cells after introducing these transgenes; however, few colonies formed, probably due to the low transfection efficiency of RCS cells and the weak transcriptional activities of Rxrb in RCS cells. Therefore, we employed another approach for RCS cells. We inserted the SV40 early promoter–puromycin resistance gene cassette at the end of the insert of the WT, 11Psub, and 11Pdel human BAC clones to generate SV40-Pur/WT, SV40-Pur/11Psub, and SV40-Pur/11Pdel (Fig. 4D). We introduced these constructs into RCS cells that were cultured in the presence of puromycin until colonies formed. Isolated colonies were picked up and transferred to separate dishes to establish stably transformed RCS cells bearing the BAC transgene constructs. We extracted total RNAs from each transfectant and performed real-time RT-PCR. PCR with human primers showed that there were no significant differences in the mean relative expression levels of human COL11A2 mRNAs to puromycin mRNAs between RCS cells bearing SV40-Pur/WT, SV40-Pur/11Psub, and SV40-Pur/11Pdel (Fig. 4D). The BAC constructs to generate WT-Pur, 11Psub-Pur, 11Pdel-Pur, and RX4sub-Pur transgenes, respectively (Fig. 4B). The BAC construction and the amounts of BAC DNAs used for transfection
four BAC transgenes were linearized by digestion with PacI restriction enzyme. Thirteen days after microinjection of transgenes, embryos were recovered, and the forelimbs on one side were dissected, frozen-sectioned, and subjected to X-gal staining. The remaining bodies were also stained with X-gal. Based on the patterns of X-gal staining, the founder mice were divided into two groups: 1) mice with X-gal staining in a variety of tissues, and 2) mice with X-gal staining mainly in cartilage. We obtained 13 founder transgenic embryos bearing the WT-LacZ transgene with positive X-gal staining. Seven embryos (54%) showed X-gal staining in a variety of tissues, whereas 6 embryos (46%) had X-gal staining mainly in the cartilage (Fig. 5B). X-Gal staining patterns of WT-lacZ transgenic founder embryos are shown in supplemental Fig. S3. To examine the possibility that the transgene constructs were degraded and only part of the BAC DNAs had integrated into the genome, we performed genomic PCR with three sets of primers that are located at the 5'-end, 3'-end, and the middle of the transgenes (Fig. 5A). The 3 sets of primers produced PCR products of the correct size from genomic DNAs extracted from all transgenic founder mice; but, no products were amplified from the genomic DNA of the non-transgenic embryos (Fig. 5C). On the assumption that BAC DNAs were integrated into the genome without degradation, the staining patterns of WT-LacZ transgenic mice suggest that the 150-kb BAC construct was not sufficient to recapitulate endogenous RXRB expression patterns. This broad domain structure of Rxrb contrasts the compactness of the Col11a2 domain, as the 2.3-kb sequence of Col11a2 was able to recapitulate expression patterns of Col11a2, even when linked to heterologous promoters.

The percentage of mice with cartilage-specific LacZ expression increased to 62 and 75% by introducing a 14-bp mutation at the 11P or RX4 sites, respectively (Fig. 5B). We cannot judge whether these increases were substantial or not. Deletion of a 507-bp stretch within the intergenic region increased to 62 and 75% by introducing a 14-bp mutation at the 11P or RX4 sites, respectively (Fig. 5B). The X-gal staining patterns of 11Pdel-LacZ transgenic founder embryos are shown in supplemental Fig. S4. These results suggest that the 507-bp deletion in the intergenic region might convert the expression domain to 90% (9 of 10 mice) (Fig. 5B). The 11Pdel-LacZ transgenic mice; but, no products were amplified from the genomic DNA of the non-transgenic embryos (Fig. 5C). On the assumption that BAC DNAs were integrated into the genome without degradation, the staining patterns of WT-LacZ transgenic mice suggest that the 150-kb BAC construct was not sufficient to recapitulate endogenous RXRB expression patterns. This broad domain structure of Rxrb contrasts the compactness of the Col11a2 domain, as the 2.3-kb sequence of Col11a2 was able to recapitulate expression patterns of Col11a2, even when linked to heterologous promoters.

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CTCF Associated with RX4, but Not with 11P in Vivo—To examine whether CTCF binds to 11P or RX4 sites in vivo, we prepared nuclei from L6 cells and performed ChIP assays with anti-CTCF antibodies. The amount of CTCF-attached DNA fragments were quantified by real-time PCR. The primers designed for RX4 amplified a product from samples immunoprecipitated with two commercially available anti-CTCF antibodies (Fig. 6). These two antibodies were raised against different domains of the CTCF protein. On the other hand, primers designed for 11P amplified only a small amount of product from
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samples immunoprecipitated with the two anti-CTCF antibodies. A substantial amount of products were amplified by primers designed for RX4 but not by primers designed for 11P from samples immunoprecipitated with anti-Rad21 antibodies. Rad21 is one of the subunits of cohesin. These results suggest that RX4 associates with CTCF and cohesin and that 11P does not associate with either CTCF or cohesin in nuclei. It has been reported that Brother of the Regulator of Imprinted Sites (BORIS) possesses an 11-zinc finger region that is highly homologous to that of CTCF, suggesting similar DNA-binding potential (35). To examine whether BORIS interacts with 11P sites in nuclei, we performed ChIP assays with anti-BORIS antibodies. Primers designed for 11P amplified a small amount of product from samples immunoprecipitated with anti-Rad21 antibodies. Primers designed for 11P amplified a small amount of product from samples immunoprecipitated with anti-BORIS antibodies that was less than the amplified amount without anti-BORIS antibodies, suggesting that BORIS did not interact with 11P sites in vivo.

DISCUSSION

RXRB and COL11A2 are located in the major histocompatibility complex region of chromosome 6p in humans. This genomic organization is preserved in mice, as Rxrb and Col11a2 are located in the major histocompatibility complex region of chromosome 17. It has not been investigated how the Rxrb and Col11a2 domains are separated.

CTCF is the only example in vertebrates where an identified protein and binding site have unambiguously been implicated in enhancer blocking activities. But it was reported that elements between T cell receptor genes and the Dad1 gene can block enhancers but do not harbor CTCF sites, suggesting the presence of CTCF-independent insulators (36). In the present study, we demonstrated that CTCF binds to the 11P sequence by EMSA. Colony assays showed that 11P was functional. However, ChIP assays showed that CTCF, Rad21, or BORIS did not bind to the intergenic region of Col11a2. It remains to be determined if 11P associates with other unknown transcriptional factors that recognize a motif similar to CTCF and regulates the transcription of Rxrb as demonstrated by colony assays. This indicates the presence of novel enhancer blocking elements required for tissue-restricted expression potentially by a previously unrecognized mechanism.

We employed BAC transgenic mice to examine the functions of the intergenic sequence between Rxrb and Col11a2 and the CTCF binding site within the Rxrb gene. The BAC clone we used contained entire COL11A2 and RXRB genes. But the WT-LacZ BAC transgene failed to recapitulate the endogenous expression patterns of RXRB. The WT-LacZ transgene directed substantial LacZ expression in the cartilage of all transgenic mice; in fact, in nearly half of the mice, the LacZ expression was most prominent in the cartilage, although the expression of endogenous Rxrb in cartilage was weak compared with its expression in other tissues. These results suggest that the Col11a2 domain affected the transcriptional activity of Rxrb in the BAC transgene, rather than that the sequences around the integrated sites directed the expression of LacZ to cartilage. These results suggest that insolation of the Rxrb promoter from the Col11a2 enhancer may be regulated by an unknown remote regulatory sequence that the BAC transgene did not cover, at least in part. The percentage of transgenic mice expressing LacZ predominantly in cartilage increased to 90 from 46% by deleting a 507-bp section of the intergenic sequence. In these mice, the LacZ expression decreased in non-cartilaginous tissues. These results suggest that a 507-bp region of the intergenic sequence may be involved in blocking the Col11a2 enhancer and silencer from affecting the Rxrb gene.

Our results suggest that a 507-bp region of the intergenic sequence is involved in the insulation of the Rxrb promoter from the Col11a2 enhancer/silencer. This 507-bp stretch spans from −530 to −90 of the transcriptional start site of Col11a2 in mice, being within the promoter of Col11a2. Indeed, elements within the 507-bp stretch contain a basal promoter (8) and cis-acting elements that increase the transcription in cartilage (27, 34) or neural tissues (27). To assay enhancer-blocking activities, typical colony assays use experimental constructs containing promoter, enhancer, and blocking elements. An element with enhancer-blocking activity interferes with enhancer-promoter communication when inserted between the enhancer and the promoter. The same element has little or no effect on transcriptional activation when present in a position flanking the promoter-enhancer pair (37). Because the 507-bp stretch contains cis-acting elements, it may affect the promoter when present in the flanking region of a promoter-enhancer pair. Thus, we did not employ typical colony assays for testing the 507-bp stretch. Instead, we performed colony assays using BAC transgenes. Deletion of the 507-bp stretch from the intergenic sequence and a substitution mutation at the 11P site decreased the promoter activities of RXRB in L6 cells, in which Col11a2 expression is suppressed, and increased the promoter activities of RXRB in RCS cells, in which Col11a2 expression is activated. These results may be consistent with the characteristics of enhancer-blocking elements; however, we cannot classify these intergenic sequences as a true insulator because we did not perform typical colony assays. The mingling of a promoter with a possible insulator reminds us of the idea that there might be considerable overlaps in the functions of promotors, enhancers, silencers, and insulators in vivo (14). Insulators may share the same bag of tricks as other regulatory elements, such as enhancers, silencers, and upstream activating sequence, which they combine in various ingenious ways to acquire specific properties.

The cartilage-derived retinoic acid-sensitive protein (Cdrap/Mia) gene is primarily expressed in cartilage and located closely flanked with housekeeping genes Snrpa and Rab4b, which are expressed ubiquitously (38). Removal of enhancer-suppressor sequences of the Cdrap gene resulted in broad tissue expression (39). These findings and our data will contribute to understanding the mechanism that regulates cartilage-specific expression from multigenic loci.

In summary, we identified a 507-bp intergenic sequence that insulates the Rxrb promoter from the Col11a2 enhancer/silencer in cell culture and transgenic mouse experiments. 11P was found to be active in cell culture experiments. Our results suggest that the intergenic sequence including 11P insulates the Rxrb promoter from the Col11a2 enhancer, probably associating with unknown factors that recognize a motif similar to CTCF.
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