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Lymphoid Enhancer Binding Factor 1 Regulates Transcription through Gene Looping

Kangsun Yun, Jae-Seon So, Arijita Jash, and Sin-Hyeog Im

Efficient transcription depends upon efficient physical and functional interactions between transcriptosome complexes and DNA. We have previously shown that IL-1β-induced lymphoid enhancer binding factor 1 (Lef1) regulates the transcription of its target genes COX2 and MMP13 in mouse chondrocytes by binding to the Lef1 binding sites located in the 3′ region. In this study, we investigated how the 3′ region-bound Lef1 regulates expression of target genes. IL-1β stimulation induced gene looping in COX2 and MMP13 genomic loci, which is mediated by the physical interaction of Lef1 with its binding partners, including β-catenin, AP-1, and NF-κB. As shown by chromosome conformation capture (3C) assay, the 5′ and 3′ genomic regions of these genes were juxtaposed in an IL-1β-stimulation dependent manner. Lef1 played a pivotal role in this gene looping; Lef1 knockdown decreased the incidence of gene looping, while Lef1 overexpression induced it. Physical interactions between the 3′ region-bound Lef1 and promoter-bound transcription factors AP-1 or NF-κB in COX2 and MMP13, respectively, were increased upon stimulation, leading to synergistic up-regulation of gene expression. Knockdown of RelA or c-Jun decreased the formation of gene loop and down-regulated cyclooxygenase 2 (COX2) or matrix metalloproteinase 13 (MMP13) transcription levels. However, overexpression of RelA or c-Jun along with Lef1 increased the looping and their expression levels. Our results indicate a novel function of Lef1, as a mediator of gene looping between 5′ and 3′ regions. Gene looping may serve to delineate the transcription unit in the inducible gene transcription of mammalian cells. The Journal of Immunology, 2009, 183: 5129–5137.

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ranscription is a continuous process in which each step is connected physically and functionally (1). Physical cross-talk between promoter and terminator regions of a gene through formation of a DNA loop serves to stabilize physical associations between elements of the transcriptional machinery and to increase transcriptional efficiency (2–4). Recently, rRNA and tRNA synthesis by RNA polymerase I and III, respectively, were shown to involve physical connections between promoter and terminator regions (5, 6). Moreover, the formation of a gene loop in RNA polymerase II-mediated transcription has been suggested in yeast (7–9) and in mammalian cells (10, 11). However, the exact molecular functions of specific transcription factors in gene looping have not yet been elucidated.

Interactions between promoter and enhancers regulate gene transcription by intrachromosomal interaction. In the immune system, T cell activation increases TNF levels by inducing intrachromosomal looping in the TNF locus (12). NFAT-containing nucleoprotein complexes mediate this interaction by inducing the association of HSS-9/HSS+3 with TNF promoter (12). CCCTC-binding factor (CTCF)5 is known to regulate the transcription of numerous target genes by modulating epigenetic states (13). At the maternal allele of the Igf2/H19 locus, CTCF binds to the unmethylated imprinting control region and mediates inactive looping by intrachromosomal interactions with promoter (14). CTCF also mediates interchromosomal association between Igf2/H19 and Wsb1/Nf1 by bridging distant DNA segments to a common transcription factor (15). The lymphoid enhancer binding factor 1 (Lef1) is a DNA-binding transcription factor that plays important roles in organogenesis, colon cancer progression (16), and cartilage degeneration (17). Notably, Lef1 is an “architectural” transcription factor that binds to DNA via its high-mobility group domain and induces a sharp bend in the DNA helix (18). The bend induced by Lef1 binding facilitates the assembly of nucleoproteins bound at nonadjacent sites (16, 19). In the center of the TCR-α enhancer, the high-mobility group domain of Lef1 bends the DNA helix and facilitates interactions between activating transcription factor/CREB and E26 transformation-specific sequence-1 (20). The Lef1-β-catenin interaction has been well characterized in the context of the canonical Wnt signaling pathway (21). β-Catenin connects with the RNA polymerase II transcription machinery through Pygo and Lgs, which interact with the N-terminal region (22–24); C-terminal regions interact with parafibromin/hyrax via polymerase-associated factor 1 (25). β-catenin is involved in the modification of chromatin structure through its association with CREB-binding protein/p300 (26) and the bralma-related gene 1-containing switch/sucrose nonfermentable chromatin remodeling complex (27).

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IL-1β is a proinflammatory cytokine that increases expression of the COX2 and MMP13 genes in arthritic cartilage (28, 29) as well as in mouse chondrocytes (30, 31). We previously reported that IL-1β regulates the expression of two transcription factors, Lef1 and its interacting transcriptional coactivator β-catenin, in primary mouse chondrocytes (17, 30, 31). In turn, these transcription factors regulate COX2 and MMP13 transcription (17, 30–32). Unusually, both COX2 and MMP13 genes feature evolutionarily conserved binding sites for Lef1 in 3′ regions and Lef1 binds to these sites in a manner dependent on IL-1β stimulation (30, 31).

To understand how Lef1 bound to the 3′ regions of the COX2 or MMP13 genes might regulate transcription of these genes in response to IL-1β, we hypothesized that Lef1 was involved in mediating long-range interactions with IL-1β-induced transcription factors that associate in a conventional manner with the 5′ regions of the COX2 or MMP13 genes. In this study, we show that NF-κB and AP-1 are induced in chondrocytes in response to IL-1β signaling and Lef1 interacts with both transcription factors in chondrocyte nuclear lysates. Furthermore, we demonstrate that the 5′ and 3′ regions of the COX2 or MMP13 genes interact in an IL-1β stimulation-dependent manner, and Lef1 plays a crucial role in this process. Lef1 depletion by small interfering RNA (siRNA) decreased the incidence of DNA looping at both the COX2- and MMP13 genes, whereas Lef1 overexpression increased the efficiency of loop formation. In addition, knockdown of NF-κB or AP-1 also suppressed the looping, whereas overexpression of those factors along with Lef1 increased formation of the DNA loop. We suggest that Lef1–mediated transcriptional regulation involves the formation of long-range DNA loops through interactions with transcription factors bound to distal regulatory elements of Lef1 target genes.

Materials and Methods

Materials and reagents

The following Abs were used: anti-Lef1, anti-phospho-c-Jun, anti-MMP13, anti-β-tubulin, and anti-lamin B, all from Santa Cruz Biotechnology; anti-β-catenin from BD Transduction Laboratories; anti-COX2 from Cayman Chemical; anti-hemagglutinin (HA) from Covance; and anti-IgG from Sigma-Aldrich. The following materials were purchased: rIL-1β from Calbiochem; control siRNA, Lef1 siRNA, RelA siRNA, and c-Jun siRNA from Santa Cruz Biotechnology and Asel, Ncol, BglII, and PsvII restriction enzymes from New England Biolabs.

Culture of mouse rib chondrocytes

Primary chondrocytes were isolated from ribs of 4-day-old imprinting control region mice and cultured as previously described (17). The chondrocytes released from cartilage were suspended in DMEM supplemented with FBS (10% [v/v]; HyClone), streptomycin (50 μg/ml), and penicillin (50 U/ml) and then plated on culture dishes at 2–4 × 10⁶ cells/cm². The cells reached confluence in 4–5 days.

Preparation of nuclear fractions, immunoprecipitation, and immunoblotting

Preparation of nuclear fractions was done as described previously (17). The resulting supernatant, containing the nuclear fraction, was retained for Lef1, RelA, and phospho-c-Jun analysis. Lamin B or β-tubulin was used as controls for preparation of nuclear fractions. Immunoprecipitation was performed as described previously (17). Briefly, after transfecting constitutively active S37A β-catenin, HA-tagged Lef1, RelA, or c-Jun or some combination of these into HEK293T cells or primary chondrocytes using Lipofectamine Plus (Invitrogen), cell lysates were prepared. The lysates were precipitated with specific Abs against HA, Lef1, RelA, or phospho-c-Jun. Then the immune complexes were collected with protein A-Sepharose beads. Immunoblotting was performed as described previously (32). The proteins were detected with specific Abs against β-catenin, HA, Lef1, RelA, phospho-c-Jun, COX2, and MMP13. Blots were developed using a peroxidase-conjugated secondary Ab (Sigma-Aldrich) and an ECL kit (Amersham Biosciences).

Chromatin immunoprecipitation (ChIP) and siRNA-coupled ChIP assays

The ChIP assay was conducted as previously described (17) using subconfluent mouse rib chondrocytes treated with IL-1β (5 ng/ml) for 1 h. The primers used in the ChIP assays were as follows: Lef1 binding site of genomic COX2, forward 5′-AATGCTGTGTTGAAGGTGTC-3′ and reverse 5′-CCTACTTGGCTGTC-3′; A, 5′-GATGCTTTTGCAGGCAGATGC-3′ (138 bp product); PsvII-restricted a, 5′-TTAAGAAATGGCCCAAGATGTTGG-3′; b, 5′-ATACCTGCAAGACGACAGAGCCA-3′; c, 5′-AAGACAGAGAGATGGAC-3′ (108 bp product).

Preparation of nuclear fractions was done as described previously (17). The primers and product size were as follows: A, 5′-CGGAGACACTTGCAAGCAA-3′ (100 bp), A, 5′-CTGGTATTTCTTCTAAGGG-3′ (231 bp product). As a loading control, PCR was performed with primers for the NF-κB binding site of genomic COX2. PCR products were resolved on 2% agarose gels or cloned into pGEM-T easy vector and sequenced. Quantitative levels of 3C assay were analyzed by real-time PCR and normalized by input DNA.

Chromatin immunoprecipitation (ChIP) and siRNA-coupled ChIP assays

The ChIP assay was conducted as previously described (17) using subconfluent mouse rib chondrocytes treated with IL-1β (5 ng/ml) for 1 h. The primers used in the ChIP assays were as follows: Lef1 binding site of genomic COX2, forward 5′-GAAGAATGCTGTGCAATGC-3′ (138 bp product); PsvII-restricted a, 5′-TTAAGAAATGGCCCAAGATGTTGG-3′; b, 5′-ATACCTGCAAGACGACAGAGCCA-3′; c, 5′-AAGACAGAGAGATGGAC-3′ (108 bp product).

Preparation of nuclear fractions was done as described previously (17). The resulting supernatant, containing the nuclear fraction, was retained for Lef1, RelA, and phospho-c-Jun analysis. Lamin B or β-tubulin was used as controls for preparation of nuclear fractions. Immunoprecipitation was performed as described previously (17). Briefly, after transfecting constitutively active S37A β-catenin, HA-tagged Lef1, RelA, or c-Jun or some combination of these into HEK293T cells or primary chondrocytes using Lipofectamine Plus (Invitrogen), cell lysates were prepared. The lysates were precipitated with specific Abs against HA, Lef1, RelA, or phospho-c-Jun. Then the immune complexes were collected with protein A-Sepharose beads. Immunoblotting was performed as described previously (32). The proteins were detected with specific Abs against β-catenin, HA, Lef1, RelA, phospho-c-Jun, COX2, and MMP13. Blots were developed using a peroxidase-conjugated secondary Ab (Sigma-Aldrich) and an ECL kit (Amersham Biosciences).
were incubated with IL-1
RelA or phospho-c-Jun Ab. PCR was performed with primers specific for the conserved NF-
binding site of
Lef1 binding site of
and reverse 5'-GTGAGCAATCTCAGCACAGT-3' (217 bp product). PCR products were resolved on 2% agarose gels with GAPDH as a loading control or PCR products were analyzed by real-time PCR normalized with L32 products.

Bioinformatic analysis
Mouse and human COX2 or MMP13 loci were aligned, and the extent of DNA sequence homology was computed with the web-based program VISTA (http://www-gsd.lbl.gov/vista) (34, 35) or ECR browser (36). Lef1 and NF-κB or AP-1 binding sites were predicted with rVISTA 2.0 (http://rvista.dcode.org) using the optimum matrix similarity.

Statistical analysis
For the statistical analysis of the data, Student’s t tests were applied on quantification experiments. A value of p < 0.05 was considered significantly different.

Results
IL-1β increases the binding of transcription factors in the 5′ and 3′ regions
We previously reported that IL-1β induces NF-κB activation in primary chondrocytes and that NF-κB is involved in the up-regulation of Lef1 transcription (17). Lef1 is required for IL-1β-mediated up-regulation of COX2 expression, and bioinformatic analysis showed that the 5′ and 3′ regions of the COX2 (Ptgs2) gene contain conserved elements for binding of NF-κB (black arrow) and Lef1 (gray arrow), respectively (Fig. 1A, top) (30, 31). These data suggested a functional cooperation between Lef1 and NF-κB for COX2 gene transcription and led us to ask whether there was a physical interaction between these two transcription factors bound to either end of the COX2 gene.

Since MMP13 expression is also up-regulated by IL-1β in a Lef1-dependent manner (31), we performed a similar analysis for the MMP13 gene. We had previously shown that, similar to the COX2 (Ptgs2) gene, the MMP13 gene also contains a conserved Lef1 binding site in the 3′ region (30, 31). However, bioinformatic analysis did not reveal conserved binding sites for NF-κB in the 5′ region of the MMP13 gene. We therefore examined the role of other inducible transcription factors that could potentially be involved in IL-1β-mediated up-regulation of MMP13. The AP-1 transcription factor, which is composed of Jun-Jun or Fos-Jun dimers, was an obvious candidate, since the 5′ region of the MMP13 gene contains a conserved AP-1 binding site (black arrow) as described previously (Fig. 1A, bottom) (37).

We first assessed the activation of NF-κB and AP-1 in IL-1β-stimulated primary mouse chondrocytes by examining the nuclear translocation of RelA and phosphorylated c-Jun (phospho-c-Jun) (38). Indeed, immunoblotting of nuclear extracts prepared from mouse chondrocytes showed a clear increase in nuclear RelA and phospho-c-Jun upon IL-1β stimulation for 1 h (Fig. 1B). Furthermore, ChIP assays showed that IL-1β stimulation of mouse chondrocytes dramatically increased the levels of RelA and phospho-c-Jun binding under physiological conditions (i.e., in intact cells) to the 5′ region of COX2 (Ptgs2) and MMP13 gene, respectively.
transcription of Lef1 target genes

Interactions of Lef1 with RelA or c-Jun up-regulate transcription factors for the regulation of Lef1 target genes. These results suggest the possibility of functional cooperation between transcription factors to a control region of the COX2 (Pgts2) gene (Fig. 1A), which contains neither a NF-κB nor AP-1 binding motif (Fig. 1C, right). We have previously reported that Lef1 binds to the conserved 3′ binding elements in the COX2 (Pgts2) and MMP13 genes by ChIP (30, 31). IL-1β stimulation increased Lef1 binding to the 3′ region of COX2 or MMP13 genes (Fig. 1D and supplemental Fig. 1B) as well as the binding of RelA or c-Jun to the 5′ region of the COX2 or MMP13 gene (Fig. 1C). These results suggest the possibility of functional cooperation between transcription factors for the regulation of Lef1 target genes.

**Interactions of Lef1 with RelA or c-Jun up-regulate transcription of Lef1 target genes COX2 or MMP13**

We next examined the interactions among Lef1, β-catenin, RelA, and c-Jun by coimmunoprecipitation assays in HEK293 cells and primary chondrocytes. Immunoprecipitates of HA-tagged Lef1 as well as RelA contained β-catenin as expected (Fig. 2A, lanes 3 and 4) (39). RelA were also coimmunoprecipitated with Lef1-HA (Fig. 2A, lane 5). Consistent with a previous report (40), immunoprecipitates of phospho-c-Jun contained Lef1-HA as well (Fig. 2B).

To confirm whether these interactions also occur in mouse chondrocytes, we performed coimmunoprecipitation assays after overexpression of Lef1 and RelA or c-Jun. Immunoprecipitates of RelA or Lef1 revealed their coexistence with Lef1 or RelA, respectively (Fig. 2C, lanes 3 and 4). In vivo association between phospho-c-Jun and Lef1 was also confirmed in chondrocytes (Fig. 2D, lanes 3 and 4).

We also assessed the functional relevance of these in vivo interactions in mouse chondrocytes, in which target gene expression was monitored by real-time PCR. When Lef1 and a constitutively active β-catenin were coexpressed with RelA in mouse chondrocytes, we observed an up-regulation of COX2 mRNA within 24 h, much greater than that elicited by Lef1/β-catenin or RelA alone (Fig. 2E). Similarly, coexpression of Lef1/β-catenin and c-Jun in primary mouse chondrocytes resulted in synergistic up-regulation of MMP13 mRNA expression within 24 h (Fig. 2F). These results suggest that physical interactions between Lef1 and RelA or phospho-c-Jun cooperatively mediate the transcription of Lef1 target genes COX2 or MMP13, respectively.

**IL-1β induces juxtaposition of the 5′ region and the 3′ region of genomic COX2 and MMP13**

The above data suggest physical as well as functional interactions between Lef1-binding 3′ elements and NF-κB or AP-1-binding 5′

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*The online version of this article contains supplemental material.*

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**FIGURE 2.** Interaction of Lef1 with RelA or c-Jun leads to up-regulation of Lef1 target genes COX2 and MMP13. A, Interactions of Lef1/β-catenin, β-catenin/RelA, and Lef1/RelA. HA-tagged Lef1, β-catenin, and RelA were transiently transfected into HEK293T cells and then RelA and Lef1-HA were immunoprecipitated (IP). Coprecipitated β-catenin or Lef1-HA was analyzed by immunoblotting (IB) with β-catenin or HA Ab. B, c-Jun with or without HA-tagged Lef1 was transiently overexpressed in HEK293T cells, and the lysates were immunoprecipitated with phospho-c-Jun Ab. Coprecipitated Lef1-HA was analyzed by immunoblotting with a HA Ab. C, Lef1 and RelA were transiently transfected into chondrocytes and then Lef1 and phospho-c-Jun were immunoprecipitated. Coprecipitated c-Jun or Lef1 was analyzed by immunoblotting with phospho-c-Jun or Lef1 Ab. The data shown in A–D are representative of three independent experiments with similar results. E, Chondrocytes were transfected with empty vector (mock) or HA-tagged Lef1 and constitutively active β-catenin (β-cat) with or without RelA for 24 h. COX2 mRNA was analyzed by real-time PCR and expressed relative to the mock sample after normalizing with mouse L32. F, Chondrocytes were transfected with empty vector (mock) or HA-tagged Lef1 and constitutively active β-catenin with or without c-Jun for 24 h. MMP13 mRNA was analyzed by real-time PCR. The data shown in E and F are the mean of three independent experiments, and error bars indicate SDs. Primary chondrocytes were isolated from ribs of 10 mice in each independent experiment.
regions of the COX2 or MMP13 genes, respectively (Fig. 1A). We therefore proceeded to evaluate our hypothesis that during IL-1β-driven transcription of the COX2 and MMP13 genes, looping interactions mediated by these transcription factors occurred between the two ends of these transcribed genes. To test the formation of these postulated gene loops, we performed chromosome conformation capture (3C) assays (8, 33). We selected the restriction enzymes AseI and NcoI for COX2 or PvuII and BglII for MMP13, since they can cut appropriate restriction sites, in-cluding the 3′ and 5′ regions of both genes (Fig. 3A). Chondrocytes were stimulated with IL-1β or left unstimulated, then treated with formaldehyde to fix their DNA conformation. Then the cross-linked DNA complexes were digested with the restriction enzyme-containing regions (Fig. 3, left second panels). In con-trast, ligated products between the more distant A and C sites under-neath the two ends of these transcribed genes. To test the interactions mediated by these transcription factors occurred between the two ends of these transcribed genes. To test the formation of these postulated gene loops, we performed chromosome conformation capture (3C) assays (8, 33). We selected the restriction enzymes AseI and NcoI for COX2 or PvuII and BglII for MMP13, since they can cut appropriate restriction sites, in-cluding the 3′ and 5′ regions of both genes (Fig. 3A). Chondrocytes were stimulated with IL-1β or left unstimulated, then treated with formaldehyde to fix their DNA conformation. Then the cross-linked DNA complexes were digested with the restriction enzyme-containing regions (Fig. 3B). The identity of all of the products was confirmed by DNA sequencing (data not shown).

In the 3C assay, ligated products can be obtained only when the restriction sites are close to one another (33). As a result, regardless of IL-1β stimulation, we obtained ligated products between the A and B in the NcoI sites of COX2 or BglII sites of MMP13 genes (Fig. 3, B and C, left top panels), presumably due to their physical proximity in the primary DNA sequence. We did not obtain ligated products between the more distant A and C sites under the same conditions (Fig. 3, B and C, left second panels). In con-trast, ligated products between the A and C sites, located at the 5′ and 3′ ends of these genes, respectively, were obtained for both genes only in the sample stimulated with IL-1β (Fig. 3, B and C, left third panels). The amount of input DNA was the same in both IL-1β-untreated and treated samples (Fig. 3, B and C, left fourth panels).

To further confirm the formation of an IL-1β-dependent loop between the 5′ and 3′ ends of the COX2 or MMP13 genes, we repeated the 3C assay with the restriction enzymes AseI or PvuII, which cut near the Lef1 binding sites (Fig. 3A, site b) as well as in the 5′ regions of both genes (Fig. 3A, site a). Again, the AseI- or PvuII-restricted products of the 3C assay were obtained only upon IL-1β stimulation (Fig. 3, B and C, right panels). As a control (41), we proceeded the 3C assay in the absence of cross-linking or li-gation. The PCR products were dramatically decreased in the absence of cross-linking (supplemental Fig. 2A) or ligase treatment (supplemental Fig. 2B). Taken together, these results demonstrate that IL-1β stimulation induces a conformational change in COX2 and MMP13 gene loci to form gene loops, such that the 3′ region which binds Lef1 is in close proximity to the 5′ region.

**Lef1 plays crucial roles in the regulation of transcription and gene looping of Lef1 target genes COX2 or MMP13**

To elucidate the role of Lef1 in the gene looping, we knocked down Lef1 expression using Lef1-directed siRNA and tested its effect on gene looping. Transfection of Lef1 siRNA into primary chondrocytes decreased Lef1 transcripts and also diminished the

![FIGURE 3. IL-1β induces juxtaposition of the 5′ and 3′ regions of genomic COX2 and MMP13. A. AseI or NcoI cleavage sites in the mouse genomic COX2 locus and PvuII or BglII cleavage sites in the mouse genomic MMP13 are illustrated: the black horizontal arrows denote the 3C primers for the AseI restriction sites (COX2) or PvuII restriction sites (MMP13); the empty horizontal arrows denote the 3C primers for the NcoI restriction sites (COX2) or BglII restriction sites (MMP13), respectively. B. Mouse chondrocytes were incubated with IL-1β for 1 h and the 3C assays restricted by NcoI or AseI were performed in the COX2 locus. C. 3C assays restricted by BglII or PvuII was performed in the MMP13 locus. Input represents products of PCR reactions using primers specific for the conserved NF-kB binding site of COX2 or PvuII to confirm that equal amounts of template DNA were present in the samples. The data shown in B and C are from one of at least three independent experiments, all of which yielded similar results. Primary chondrocytes were isolated from ribs of 10 mice in each independent experiment.

![FIGURE 4. Lef1 plays a crucial role in the gene looping. A. Chondrocytes were transfected with Lef1 or control siRNA and then stimulated with IL-1β (left). Chondrocytes were transfected with empty or Lef1 expression vector for 24 h (right). The mRNA levels of Lef1, COX2, and MMP13 were analyzed by RT-PCR. GAPDH was analyzed as a loading control. B. Left, chondrocytes were transfected with Lef1 or control siRNA for 24 h and IL-1β was further added for 1 h. The 3C assay was performed with AseI restriction for COX2 or PvuII restriction for MMP13. Right, Chondrocytes were transfected with empty or Lef1 expression vector for 24 h and the 3C assay was performed with AseI restriction for COX2 or PvuII restriction for MMP13. Input represents PCR products of a conserved NF-kB binding site of COX2. C. After incubation with IL-1β, physiological interaction of Lef1 and RelA or c-Jun were shown by ChIP assays: left (upper), RelA or Lef1 was immunoprecipitated and PCR was performed with primers specific for the conserved Lef1 or NF-kB binding site of COX2. Right (upper), phospho-c-Jun or Lef1 was immunoprecipitated and PCR was performed with primers specific for the conserved Lef1 or AP-1 binding site of MMP13. Input denotes the PCR products obtained with genomic DNA without immunoprecipitation. Left (lower), Lef1 was immunoprecipitated and PCR was performed with primers specific for the conserved NF-kB binding site of BMP2. Right (lower), c-Jun was immunoprecipitated and PCR was performed with primers specific for the conserved Lef1 binding site of cyclin D1. Input denotes the PCR products obtained with genomic DNA without immunoprecipitation. The data shown in A–C are from one of at least three independent experiments that yielded similar results. Primary chondrocytes were isolated from ribs of 10 mice in each independent experiment.**
levels of COX2 and MMP13 gene expression (Fig. 4A, left panels) and protein levels (supplemental Fig. 5A) in response to IL-1β stimulation as expected (supplemental Fig. 3A for quantitative analysis). In a 3C assay, Lef1 depletion also diminished the efficiency of gene looping assessed by formation of the ligation product between sites a and b in AseI restriction for COX2 or PvuII restriction for MMP13 (Fig. 4B, left panels, and supplemental Fig. 3B for quantitative analysis). We also evaluated the effect of Lef1 overexpression, in the absence of IL-1β stimulation, on transcript levels and gene looping of COX2 and MMP13. Cells transfected with Lef1 showed an increase in both gene expression and 3C product, whereas cells transfected with empty vector showed neither (Fig. 4, A and B, right panels, and supplemental Fig. 3, A and B, for quantitative analysis). Together, these data indicate a crucial role of Lef1 in gene looping and also suggest that gene looping is correlated with active transcription.

We next asked whether the IL-1β-mediated gene looping could be mediated by the physical interaction of 3’ region-bound Lef1 with 5’ region-bound NF-κB or AP-1 in COX2 and MMP13 loci, respectively. Accordingly, we performed ChIP analysis. After immunoprecipitation with a RelA or phospho-c-Jun Ab, PCR was performed with primers specific for each Lef1 binding site in the 3’ regions of the COX2 or MMP13 genes. Conversely, after immunoprecipitation with a Lef1 Ab, PCR was performed with primers specific for the 5’ NF-κB or AP-1 binding sites of the COX2 or MMP13 genes, respectively. The amount of Lef1-bound DNA on the 3’ region at the COX2 locus was enriched after IL-1β treatment, as shown following immunoprecipitation with a RelA Ab (Fig. 4C, upper left top, and supplemental Fig. 3C for quantitative analysis). Likewise, NF-κB (RelA)-bound DNA at the promoter was also increased after immunoprecipitation with a Lef1 Ab (Fig. 4C, upper left middle, and supplemental Fig. 3C for quantitative analysis). We observed similar results from ChIP analysis in the MMP13 locus. The ChIP products in the MMP13 genomic locus were increased after IL-1β stimulation (Fig. 4C, right, and supplemental Fig. 3C for quantitative analysis). To show the specificity of Lef1 binding to the RelA-binding 5’ region at the COX2 locus or c-Jun binding to Lef1-binding 3’ region at the MMP13 locus, ChIP assay was performed. ChIP analysis in Bone morphogenetic protein 2 (BMP2) or cyclin D1 loci was also performed as negative controls (Fig. 4C, bottom). The BMP2 gene contains a NF-κB-binding element in the −838/-829 region (42). The Cycin D1 gene has a Lef1 binding site in the −81/-73 region (43). After immunoprecipitation with a Lef1 or c-Jun Ab, PCR was performed with primers specific for the NF-κB (RelA) or Lef1 binding site of the BMP2 or cyclin D1 gene, respectively. Lef1 did not interact with the NF-κB binding site of the 5’ region in the BMP2 gene, and c-Jun did not bind to the Lef1 binding sites of the 5’ region in the cyclin D1 gene, respectively. Taken together, these data suggest that the 3’ region-bound Lef1 interacts with the RelA-binding 5’ region of the genomic COX2 locus. In the MMP13 transcriptions, the 3’ region-bound Lef1 physiologically cooperates with the AP-1-binding 5’ region in an IL-1β stimulation-dependent manner. Indeed, co-overexpression of RelA and Lef1/β-catenin resulted in synergistic up-regulation of COX2 expression (Fig. 2E). MMP3 expression was also increased by co-overexpression of c-Jun and Lef1/β-catenin (Fig. 2F).

Knockdown of RelA or c-Jun reduces Lef1 binding to the 5’ region of COX2 or MMP13

Interaction of Lef1 with the 5’ region-bound RelA or c-Jun in COX2 or MMP13 locus enhanced their transcription (Fig. 2) through gene looping (Figs. 3 and 4). To further elucidate the role of RelA or c-Jun in these associations, we analyzed their knock-down effect on COX2 or MMP13 transcription and gene looping. Transfection of RelA siRNA or c-Jun siRNA into primary chondrocytes decreased the levels of COX2 or MMP13 gene expression and protein levels, respectively, (Fig. 5A and supplemental Fig. 5, B and C). Lef1 siRNA treatment reduced gene looping between the 5’ region and 3’ region of COX2 or MMP13 genes (Fig. 4B). We further tested whether treatments of siRNAs for RelA, RelA, or c-Jun directly diminished the physical interaction of the 5’ region binding RelA or c-Jun with the 3’ region binding Lef1 in COX2 and MMP13 loci, respectively. After Lef1 siRNA treatment and then immunoprecipitation with RelA or phospho-c-Jun Ab, PCR was performed with primers specific for the Lef1 binding site in the 3’ regions of the COX2 or MMP13 genes. As expected, Lef1 siRNA treatment diminished the interactions between 5’-bound transcription factors and the Lef1-bound 3’ region (Fig. 5B and supplemental Fig. 4A for quantitative analysis). Next, we assessed the effect of siRNA treatment for RelA or c-Jun on these interactions. After RelA siRNA or c-Jun siRNA treatment, ChIP was performed with a Lef1 Ab. Enriched DNA was then analyzed by PCR with primers specific for the 5’ NF-κB or AP-1 binding sites of the COX2 or MMP13 genes, respectively. Treatment with RelA siRNA or c-Jun siRNA reduced NF-κB or AP-1-bound DNA levels at the 5’ region (Fig. 5C, top, and supplemental Fig. 4B for
FIGURE 6. Crucial role of Lef1 and its binding partners in gene looping. A, Chondrocytes were transfected with siRNA for RelA, c-Jun, or control (Cont) siRNA and then stimulated with IL-1β. Left, The 3C assay was performed with Asel restriction for COX2 or PvuII restriction for MMP13. Input denotes the PCR products obtained with genomic DNA without immunoprecipitation using primers specific for the conserved NF-κB binding site of COX2. Right, The 3C values were analyzed by real-time PCR using 3C primer and expressed relative to control siRNA after normalizing with input DNA. B, Upper, chondrocytes were transfected with Lef1 or RelA expression vector for 24 h. The 3C assay was performed with Asel restriction for COX2. Lower, The 3C values were analyzed by real-time PCR using 3C primer and expressed relative to control (empty vector). C, Upper, Chondrocytes were transfected with Lef1 or c-Jun expression vector for 24 h. The 3C assay was performed with PvuII restriction for MMP13. Lower, The 3C values were analyzed by real-time PCR using 3C primer and expressed relative to control. The data shown in A–C are from one of at least three independent experiments that yielded similar results. The error bars show the SDs of the PCR performed in triplicate. Primary chondrocytes were isolated from ribs of 10 mice in each independent experiment.

quantitative analysis). Taken together, these data suggest that the 3′ region-bound Lef1 interacts with the 5′ region-bound RelA or c-Jun on the COX2 or MMP13 locus, respectively.

Interaction of Lef1 with RelA or c-Jun cooperatively regulates gene looping

Knock-down of Lef1, RelA or c-Jun suppressed the expression of Lef1 target genes in chondrocytes, and reduced the association between 5′-bound RelA or c-Jun and 3′-bound Lef1 (Fig. 5). To test the role of RelA or c-Jun in the formation of gene loops, 3C assays was performed after siRNA treatment for RelA or c-Jun. We used the restriction enzymes Asel or PvuII, which cut near the RelA or c-Jun binding sites of COX2 or MMP13, respectively (Fig. 3A). Knock-down of RelA reduced the efficiency of gene looping assessed by formation of the ligation product between sites a and b in Asel restriction for COX2 (Fig. 6A). Similarly, c-Jun siRNA suppressed the loop formation as assessed by PvuII restriction for MMP13 (Fig. 6A). These data showed that gene looping in COX2 or MMP13 is also mediated by RelA or c-Jun as well as Lef1.

The above data also suggest that intrachromosomal interactions are mediated through the interaction of transcription factors binding to the 5′ and 3′ region of COX2 or MMP13 genes. To confirm the cooperative role of Lef1, RelA or c-Jun in the formation of a loop between the 5′ and 3′ region of the COX2 or MMP13 genes, chondrocytes were transfected with expression vectors of Lef1, RelA or c-Jun in the absence of IL-1β stimulation. In the COX2 gene locus, loop formation was increased by over-expression of Lef1 or RelA. In addition, this loop formation was further increased by over-expression of Lef1 and RelA together (Fig. 6B), which mediated additive up-regulation of COX2 transcription (Fig. 2E). Likewise, gene looping between 5′ and 3′ region of MMP13 was increased by coexpression of Lef1 and c-Jun together compared with individual over-expression of either alone (Fig. 6C). This result was also in accordance with expression pattern of MMP13 (Fig. 2F). Taken together, these results demonstrate that IL-1β stimulation induces intrachromosomal interaction in COX2 or MMP13 gene loci to form gene loops, which is mediated by juxtaposition between the Lef1-binding 3′ region and the RelA or c-Jun-binding 5′ region. Collectively, these results suggest the critical role of transcription factors in gene looping and the close correlation of gene looping with active gene expression.

Discussion

COX2 and MMP13 are critical molecules involved in the immune response and inflammation of rheumatoid arthritis and osteoarthritis. They are primarily produced by chondrocytes of arthritic cartilage. COX2 catalyzes the production of PGE₂, which mediates joint inflammation. MMP13 is a proteolytic enzyme that degrades the extracellular matrix of cartilage tissues (44). We previously reported that Lef1 is highly expressed in osteoarthritic cartilage and induced by IL-1β in mouse chondrocytes (17). We also showed that proinflammatory cytokine IL-1β up-regulates COX2 and MMP13 levels by increasing Lef1 binding to the 3′ regions of target genes (30, 31). In this study, we further elucidated how the 3′ region-binding Lef1 regulates its target genes COX2 and MMP13 in chondrocytes.

We have demonstrated that Lef1-mediated regulation of target genes in chondrocytes COX-2 or MMP13 correlates with the looping contact of the Lef1-binding 3′ region with 5′ regions which
bound by the inducible transcription factors NF-κB or AP-1, respectively. It has been suggested that gene looping is associated with transcription reinitiation, a cyclic process of RNA synthesis in active genes (5). To maintain the accelerated transcription rate, RNA polymerases and transcription factors need to be recycled (45). The formation of a gene loop, in which both ends of the transcription unit are juxtaposed, could physically stabilize the active transcriptional machinery and direct new rounds of transcription. This would eventually result in an accelerated transcription rate and/or an increase in transcriptional efficiency (5).

The formation of gene loops in RNA polymerase II-mediated transcription has been previously reported. The incidence of gene looping was determined by the phosphorylation status of RNA polymerase II (8, 10). Cleavage and polyadenylation factor 3’-end processing machinery also functions in RNA polymerase II-dependent gene looping (7). TFIIIB is suggested as another connector of gene looping (9). Our study suggests that an architectural transcription factor, Lef1, is associated with the gene looping in mammalian cells; 3’ region-bound Lef1 induced gene looping through the interaction of 5’ region-bound transcription factors NF-κB or AP-1. In addition, our results also suggest that gene looping in mammalian cells is involved in the early stages of transcriptional activation (8), since transcription factors usually bind to DNA before formation of the preinitiation complex formation, necessitating recycling.

Binding of Lef1 to its consensus DNA sequence induces a sharp bending in DNA structure (46). Interactions of 3’ region-bound Lef1 with other factors can direct the bending of DNA. Most known transcription factors bind to promoter regions. Therefore, the bent DNA in the 3’ region will result in the formation of a gene loop by the turn toward the promoter bound by diverse transcription factors. Such a role of the 3’ region-bound Lef1 is consistent with the formation of a gene loop which is mediated by interaction with other transcription factors. Indeed, in immune cells, Lef1/β-catenin regulate TCR-α and HIV-1 transcription in a chromatin-dependent manner by recruiting chromatin remodeling complexes (47). β-Catenin, a well-known Lef1-binding transcriptional coactivator, plays crucial roles both in connecting DNA to RNA polymerase II transcription machinery and in chromatin remodeling; Pygo and Lgs link β-catenin to the RNA transcription machinery (22–24) and parafibromin/hyrax is physically connected to the C-terminal of β-catenin with platelet-activating factor 1 (25). These functions of Lef1/β-catenin might be appropriate for gene looping and thus recycling of RNA transcription machinery. Thus, there might be cooperative roles between Lef1/β-catenin and remodeling factors for inducing gene looping. Initiation of local DNA bending by Lef1 might lead to the formation of a gene loop through chromatin remodeling, which is mediated by β-catenin-interacting proteins. Taken together, locus-specific looping mediated by Lef1 binding can provide the mechanism for specific and efficient transcription of Lef1 target genes. Furthermore, the conservation of the Lef1 binding site in the 3’ region might be evolutionarily developed to facilitate gene looping.

Stimulation induces more efficient transcription by inducing the interactions between transcription factors through looping mechanisms. IL-1β stimulation up-regulated the protein level of Lef1 or β-catenin in chondrocytes (30). Interestingly, at the same time, we also observed an increase of NF-κB or AP-1 translocation into the nucleus upon IL-1β stimulation (Fig. 1B). These imply that IL-1β stimulation may facilitate the cooperative interaction between diverse transcription factors, resulting in efficient expression of target genes. Interactions between proteins bound at nonadjacent sites mediate bending or looping of the DNA and generate the higher-order nucleoprotein complex (48). Architectural proteins induce the formation of these complexes by bending DNA to facilitate the interactions between cis-acting elements. Widely separated cis-acting regulatory elements at gene loci evidence the functional interaction between regulatory elements (49) and provide a mechanism for efficient transcription of genes. Stimulation-dependent DNA looping mediated by activated transcription factors should be efficient in terms of energy expenditure and rate of transcription. We showed the pivotal role of Lef1 in gene looping of COX2 or MMP13 under IL-1β signaling. Besides Lef1/β-catenin, NF-κB, and AP-1, other transcription factors may also be involved in gene looping following IL-1β stimulation. Indeed, knockdown of Lef1 (Fig. 4B), RelA, or c-Jun (Fig. 6A) decreased the incidence of gene looping while overexpression of them induced looping (Figs. 4B and 6, B and C). In addition, overexpression of Lef1 along with its 5’ region-binding partner RelA or c-Jun additively up-regulated gene looping (Fig. 6, B and C) as well as up-regulation of Lef1 target genes COX2 and MMP13 (Fig. 2, E and F). However, further studies are needed to determine how the association between Lef1 and RelA or c-Jun is mediated for inducing a gene loop.

Interactions between DNA-bound transcription factors direct formation of an enhanceosome, which induces transcriptional synergy throughout the gene from the promoter to the terminator (50, 51). Multiple interactions between transcription factors may strengthen the assembly of enhanceosomes and facilitate the formation of gene loops. As suggested in yeast, RNA polymerase II-mediated transcription may be facilitated by gene looping. Long-distance associations between regulatory elements may play an important role in eukaryotic gene regulation. Inactivation or detachment of transcription factors from enhanceosomes could destabilize gene loops, leading to termination of transcription. Therefore, the transcription factors involved in gene looping may form the mechanism whereby transcription is switched on and off. Investigation of 3’-bound transcription factors may reveal further diverse gene looping systems.

In conclusion, Lef1 mediates COX2 and MMP13 gene looping during RNA polymerase II-mediated transcription in mammalian cells. Formation of a DNA loop as a transcription unit could increase the efficiency of RNA polymerase II-mediated transcription by increasing the transcription rate while conserving energy, an effect that could have particular significance in the mammalian system, where the typical transcription unit is relatively long. Gene looping may constitute a mechanism for inducible transcriptional regulation in mammalian cells and 3’ region-bound transcription factors may function as connectors forming integrated transcription units.

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