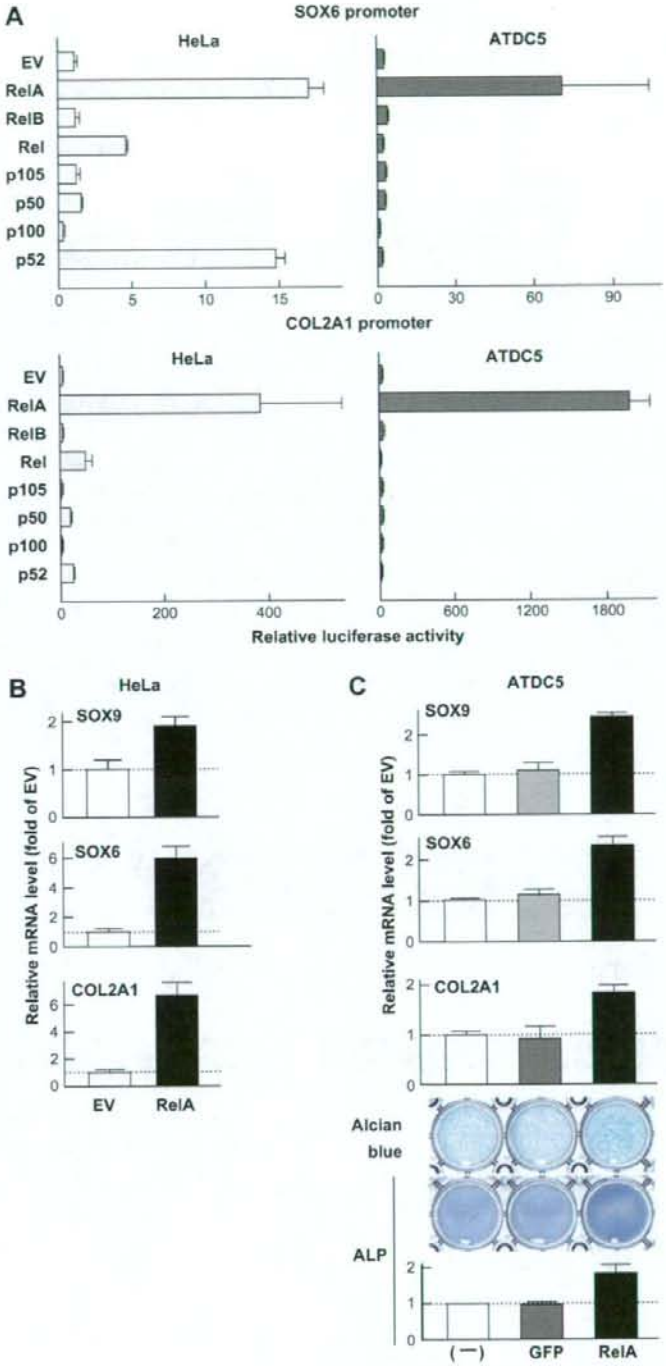

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differentiation medium [Fig. 6(C)]. In addition to the mRNA levels of SOX9, SOX6, COL2A1, the chondrogenic differentiation markers Alcian blue and ALP activity were stimulated by the RelA overexpression. The raw values of SOX9, SOX6, and COL2A1 mRNA levels normalized by G3PDH in the control HeLa cells were 1999 ± 29, 486 ± 90, and 21 ± 2 copies/μg of total RNA [Fig. 6(B)], while those in the control ATDC5 cells were 118 ± 8, 356 ± 11, and 6983 ± 214 copies/μg of total RNA, respectively [Fig. 6(C)], indicating that HeLa cells express negligible level of COL2A1 despite higher levels of SOX9 and SOX6 than ATDC5 cells. These results indicate that RelA functions as an inducer of chondrogenic differentiation, probably *via* the SOX9 transactivation.

Discussion

The present comparison of the promoters between human and mouse SOX9 genes found an NF-κB family member RelA to be a transcription factor of SOX9. We further identified an NF-κB binding motif around −250 bp as the core region responsive to RelA in the human SOX9 proximal promoter. In several previous studies, the sequences of the mouse and human SOX9 genes were compared and functional analyses of the SOX9 promoter were made. Morishita *et al.* identified a 30-bp region in the first intron as an ATDC5-specific enhancer, although the related transcriptional regulation remains unclear[28]. Kanai and Koopman showed that the region between −193 and −73 bp is essential for the sex- and tissue-specific expression of the mouse SOX9 gene[29]. These findings were consistent with those of a later study by Colter *et al.* on the human SOX9 promoter in which activity decreased when a deletion was made past position −172 bp[30]. In the region, they identified two CCAAT motifs that are important for the SOX9 promoter activity in chondrogenic cells. In the present deletion analysis of the luciferase assay, these motifs are located between −127 and −91 bp and between −91 and −50 bp [the top schema in Fig. 4(B)]. Although the transactivity induced by RelA did not differ between the regions, the baseline activity without the RelA stimulation was actually decreased between them [Fig. 4(A)]. This confirms the regulation of SOX9 transactivity by C/EBP proteins through its interaction with the two CCAAT motifs located more proximal to the present NF-κB motif in the SOX9 promoter. In fact, C/EBP proteins showed potent transactivation of SOX9 in both HeLa and ATDC5 cells (Fig. 2). More recently, the same group reported that the human SOX9 proximal promoter is also regulated by the cyclic-AMP response element binding (CREB) protein and Sp1[31]. In the present deletion analysis, the binding motifs are located between −202 and −128 bp, which we identified as the proximal element [the top schema in Fig. 4(B)]. Here again, the baseline transactivity was decreased as well as the RelA-induced activity. In the tandem-repeat experiments, the baseline transactivity was increased dependent on the repeat number of the proximal element, though not as strongly as that of the distal element (−202/−128) under the RelA stimulation [Fig. 4(B)]. Interestingly, the decrease in the deletion analysis and the increase in the tandem-repeat analysis of the proximal element were equivalently seen between the presence and absence of the RelA stimulation, while those of the distal element were apparent only under the RelA stimulation (Fig. 4). These indicate that the identified NF-κB motif in the distal element is specific to the RelA stimulation, while the proximal element including the CREB and Sp1 motifs functions as a basal regulatory region in the SOX9 proximal promoter.

The RelA overexpression enhanced the promoter activities and the endogenous mRNA levels of SOX6 and COL2A1 in HeLa and ATDC5 cells (Fig. 6). These may be at least partly mediated by the RelA effect on the SOX9 transactivation, since SOX9 is a crucial transcriptional activator of SOX6 and COL2A1[4–6,10]. Although the RelA overexpression enhanced chondrogenic differentiation shown by Alcian blue staining and ALP activity in differentiated ATDC5 cells after the stimulation by ITS and Pi [Fig. 6(C)], this was not reproducible in undifferentiated ATDC5 cells without the stimulation (data not shown). Considering that the promoter assays were performed in ATDC5 cells without the differentiation stimulation, there is a discrepancy between endogenous mRNA levels and exogenous promoter activities of SOX9, SOX6 and COL2A1 in the RelA actions on undifferentiated ATDC5 cells. This might be due to post-transcriptional negative regulation that was specific to endogenous mRNAs or the chromatin regulation occurring only in a genomic context, which are specific to undifferentiated ATDC5 cells. In fact, EMSA using nuclear extracts from ATDC5 cells revealed that complex formation with the NF-κB probe was much stronger in extracts from differentiated cells than in those from undifferentiated cells [Fig. 5(B), lanes 14 and 15]. Contrarily, our previous study has shown that the overexpression of SOX trio or the SOX9 alone potently stimulated chondrogenic differentiation even from non-chondrogenic cells[14], indicating that the RelA may not induce sufficiently high SOX9 levels to force chondrogenic differentiation in the absence of additional stimulation.

Although the present study focused on a region within 1 kb of the 5′-end flanking region of the SOX9 gene and identified RelA as the potent transactivator of the limited region, there are surely more distant regions that are critical for the SOX9 expression. The fact that translocation breakpoints in campomelic dysplasia patients have been mapped 50 kb- or more distant from SOX9[13,32] indicates the large genomic environment regulating SOX9 expression *in vivo*. In mice as well, suppression of limb outgrowth by the blockage of the NF-κB pathway was shown to be due to defects in fibroblast growth factor (FGF) signal which caused a failure in mesenchymal–epithelial communication, rather than to a defect in chondrogenesis[20,33]. In addition to the abovementioned signals that directly

Fig. 6. (A) Promoter activities of SOX6 and COL2A1 by the NF-κB family members in HeLa and ATDC5 cells. The cells were co-transfected with the luciferase-reporter construct containing the SOX6 promoter fragment (−517 to IVS1 + 23 in the human SOX6 gene) or the COL2A1 promoter fragment (four repeats of the 49 bp SOX9 enhancer and the basal promoter from −183 to +23 bp in the human COL2A1 gene), and the NF-κB family factors or the control EV. Data are shown as means (bars) ± S.E.M. (error bars) of relative luciferase activity (the ratio of the firefly activities to the renilla activities) for 4 wells/group. (B) mRNA levels of endogenous SOX9, SOX6, and COL2A1 determined by real-time RT-PCR in HeLa cells that were transiently transfected with RelA or the control EV. Data are shown as means (bars) ± S.E.M. (error bars) of relative mRNA level as compared to the EV-transfected cells for 3 wells/group. (C) mRNA levels of endogenous SOX9, SOX6, and COL2A1, Alcian blue staining, ALP staining and activity (relative to control) in stable lines of ATDC5 cells retrovirally transfected with RelA or the control green fluorescence protein (GFP) and in non-transfected parental cells (−) after culture for 3 weeks with ITS and 2 d with Pi. The relative mRNA data are shown as means (bars) ± S.E.M. (error bars) as compared to the non-transfected parental cells (−) for 3 wells/group.

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activate the putative motifs such as CCAAT, CREB, and Sp1 within the 1 kb promoter, there are several pathways known to induce the SOX9 expression. FGFs have been shown to up-regulate SOX9 mRNA expression in chondrocytes through a MAP kinase pathway[34]. Bone morphogenetic proteins and hedgehog family members enhance SOX9 expression under certain conditions, while retinoic acid exhibits mixed results[8,35–37]. Hence, we surmise that RelA is not the principal transactivator of SOX9, but is a member of complicated molecular network for the transactivation. Addition of other signals to RelA will be needed to achieve strong SOX9 induction and efficient chondrogenic differentiation.

Proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF)-α are known to be representative ligands for the NF-κB signal[38–41]. A previous report showed that IL-1 and TNF-α caused a suppression of SOX9 expression in chondrogenic cells, which may explain the deleterious role of the cytokines in cartilage degenerative disorders such as rheumatoid arthritis and osteoarthritis[42]. Interestingly, the report indicated that the SOX9 suppression is at least partly mediated by the NF-κB signal induced by the cytokines. Furthermore, a recent study showed that silencing of IKKβ enhanced the accumulation of glycosaminoglycan in conjunction with increased SOX9 expression in human osteoarthritis chondrocytes[43]. These indicate the down-regulation of SOX9 by the NF-κB signal, which seems contradictory to the present results showing the positive relationship between them. A previous study, however, has shown that the SOX9 suppression by NF-κB occurs not at the transcriptional level, but at the post-transcriptional level through the RNA sequence-dependent mechanism[38]. At the transcriptional level as well, there may be pathways other than NF-κB in the SOX9 suppression by the proinflammatory cytokines, since the human promoter study above has shown that IL-1 down-regulated SOX9 promoter activity through a reduction of Sp1 binding to the proximal promoter in chondrocytes[31]. Hence, the NF-κB and SOX9 signals may regulate chondrogenic differentiation and skeletal development *via* complicated mechanisms by various kinds of interactions with each other.

Regarding RelA, to date a description of the *in vivo* function has been limited to the embryonic lethality of the homo-knockout mice. Since a recent report demonstrated that Nkx3.2 supports chondrocyte survival by activating RelA *via* a ligand-independent mechanism[44], RelA might possibly function to maintain the chondrogenic phenotype through constitutive activation of SOX9. Further understanding of the molecular network related to the RelA/SOX9 axis will lead to elucidation of the mechanism underlying chondrogenic differentiation and cartilage formation under physiological and pathological conditions.

Conflict of interest

The authors declare that they have no conflicts of interest.

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