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JunB mediates enhancer/promoter activity of *COL1A2* following TGF-β induction

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ABSTRACT

Transcriptional control of the genes coding for collagen type I is regulated by a complex interaction between a distal enhancer and a proximal promoter. In this study, we have dissected the molecular mechanism of this interaction by defining a specific sequence within the enhancer that respond in fibroblasts to transforming growth factor-β (TGF-β). We show that TGF-β activates *COL1A2* gene via a non-canonical (Smad-independent) signalling pathway, which requires enhancer/promoter co-operation. This interaction involves exchange of cJun/Jun B transcription factor occupancy of a critical enhancer site resulting in the stabilization of enhancer/promoter coalescence. Moreover, using transgenesis, we show that interference in this mechanism results in the abolition of *COL1A2* fibroblast expression *in vivo*. These data are therefore relevant to the control of collagen type I *in vivo* both in embryonic development, in adult connective tissue homeostasis, and in tissue repair and scarring pathologies.

INTRODUCTION

Collagen type I is an integral structural component of the extracellular matrix (ECM) and crucial for the formation of connective tissues. Collagen type I is made up of two polypeptides, COL1A1 and COL1A2, which are transcribed by two separate genes. The control of these genes requires precise regulation and organization at the transcriptional level. Current knowledge of transcriptional regulation of *COL1A2 in vivo* has been obtained primarily from studies using promoter sequences driving transgene expression in mice. Transgenic mice harbouring *COL1A2* proximal promoter alone (~400 bp upstream of transcription start site) exhibit generally low levels of activity in late gestation embryos (E15.5) and express the transgene in only a small subset of mesenchymal cells (1). The search for other collagen type I transcriptional regulatory sequences led to the identification of the 'far upstream enhancer' region located 21.8 to –18.8 kb upstream of the start site of transcription of the human *COL1A2* gene. When this enhancer region is cloned upstream of the proximal promoter, it increased the levels of β-galactosidase reporter gene significantly in almost all mesenchymal tissues where the endogenous *COL1A2* is expressed (1). High degree of homology was found between the human and murine enhancers (2). In addition to the absolute requirement of the enhancer to govern temporal and tissue-/cell-specific expression of collagen *in vivo*, the presence of the enhancer has also been shown to be essential for the re-activation of collagen type I in adult mice during repair (3), wound healing (4) and in pathologies such as scarring and fibrosis (4–7).

Transforming growth factor-β (TGF-β) has a role in controlling the lineage-specific expression of collagen type I genes, as demonstrated by the high degree of temporal and spatial correlation between activation of collagen type I genes and the presence of TGF-β in the extracellular environment (8). TGF-β has been shown to exert its effect on the *COL1A2* gene through a TGF-β response element (TbRE) located between nucleotides −313 and −250 upstream of the transcriptional start site in the human *COL1A2* proximal promoter (9,10). This TbRE sequence is essential for the canonical Smad3/4-mediated TGF-β response of this promoter and was shown to act synergistically with three Sp1 sites upstream (11,12). An activator protein-1 (AP-1) site (−250 bp), downstream of the triple Sp1 sites, has also been shown to play a role in TGF-β transcriptional activation of *COL1A2 in vitro* (13,14).

In adult tissues, TGF-β is known to be involved in the regulation of many cellular activities, including wound

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healing, tissue repair and remodelling. The persistence of TGF-β and the activation of its downstream targets genes have been the focus of many investigations in biological settings (15–19). TGF-β expression also co-localizes with activated fibroblasts responsible for most of type I collagen deposition in fibrotic diseases (20,21). In addition to fibroblasts, TGF-β can control collagen type I expression by acting on other cells, such as epithelial cells, and promoting epithelial-to-mesenchymal transition, thus contributing to the fibroblast pool in kidney, lung, heart and liver fibrosis (22–26).

Since COL1A2 enhancer is critical in controlling the level and lineage-specific expression of collagen in development and in adult tissues, we investigated whether the enhancer is TGF-β responsive. We report for the first time the presence a novel TbRE located far upstream in the enhancer of the human *COL1A2*, which works independently of the previously described TbRE in the proximal promoter. This enhancer element activates transcription via a non-canonical (Smad-independent) pathway involving an exchange of AP-1 family partner occupancy at a specific site within the enhancer. We show the long-range interaction with a critical proximal promoter site and that mutation of this site results in the abolition of *COL1A2* expression in fibroblasts using reporter gene expression *in vivo*.

MATERIALS AND METHODS

Cell culture

NIH3T3 mouse embryonic fibroblasts were cultured in (Gibco BRL), 10% fetal calf serum (FCS) (Gibco, Grand Island, NY, USA). Prior to TGF-β treatment (2.5 ng/ml), the cells were serum-starved for 12 h. Primary Smad-3-null fibroblasts were a kind gift from Dr. C. Flanders (NIH, USA).

Primary human dermal fibroblasts were explanted from the skin biopsies taken with permission from healthy control subjects. Fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM), 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and cultured in a humidified atmosphere of 5% CO_2 in air. Fibroblasts were sub-cultured at confluence and used between passages 2 and 5.

DNA constructions

The human collagen far upstream enhancer region has been previously described by Antoniv *et al.* (1). Briefly, construct –378 LAC in the β-gal-Basic vector (CLONTECH, Palo Alto, CA, USA) was derived from the −378 COL1A2/LUC plasmid. Constructs were engineered by subcloning various upstream sequences 5′ of the −378 promoter (see Supplemental Data). The HSV-thymidine kinase promoter (pRL-TK) (Promega) was used to provide a heterologous promoter to replace the COL1A2 proximal promoter (see Supplemental Data).

Smaller enhancer constructs F1-pLac (–20.7 kb to −20.45 kb), F2-pLac (−20.17 kb to −19.94 kb) and F3-pLac (−18.96 kb to −18.8 kb) containing sequences which include DNAse footprints (1) were made by polymerase chain reaction (PCR) amplification and insertion into the pLAC vector.

Mutagenesis

We have followed the recommendation of Risse *et al.* (27) in the mutagenesis of the AP-1 binding in the minimal promoter and in the enhancer region (see Supplemental Data).

Transient transfections

Transient transfection assays were performed using the FuGENE™ 6, according to the manufacturers' instructions. All transfections were performed in triplicate and repeated on more than three separate occasions. Statistical analysis of data was performed using the Student's *t*-test.

β-Galactosidase and luciferase assays

The activities of reporter genes, β-galactosidase and luciferase, used in the transient transfection assays were measured using the Dual-Light® Chemiluminescent reporter gene assay system (Tropix), according to the manufacturers' instructions.

Inhibition using small interfering RNA (siRNA)

Jun B siRNA target sequences were obtained from Dharmacon (Jun B siGenome® Smartpool reagent M-003269) and were used according to the manufacturers' instructions. Transfection into explanted human dermal fibroblasts was achieved using the *Trans*IT-TKO® transfection reagent (Mirus, MIR2150), according to the manufacturers' instructions. The siRNA was transfected at two concentrations (5 nM and 20 nM). Control siRNA was also used at the 20-nM concentration (Dharmacon, siCONTROL, D-001210-01-20)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and western blot analysis

Fibroblasts were seeded (2.5×10^5 cells per well) for 48 h. The cells serum were starved for 12 h and treated with or without TGF-β1 (R&D Systems Inc. UK), at 4 ng/ml, for a further 24 h. The cells were lysed with Laemmli buffer and analysed by SDS–PAGE, followed by Western blotting. The antibodies c-jun Jun B, Jun-D from (Santacruz), and anti-type I collagen antibody (Southern Biotechnology Associates Inc, Birmingham, AL, USA) were used. A chemilluminescence kit (Amersham) was used to develop the western blots.

Electrophoretic mobility shift assays (EMSAs)

The nuclear extracts were used in EMSA according to the standard protocol described in the commercially available EMSA kit (Promega). Super-shift assays were carried out using specific antibodies raised against phospho-c-Jun, Jun B, Sp1, EGRF (Santa Cruz).

Chromatin immunoprecipitation (ChIP) assays

ChIP assay was performed on primary human dermal fibroblasts or on NIH3T3 fibroblasts that were treated

or not treated with TGF-β using a commercially available kit (Upstate), according to the manufacturers' instructions. Briefly, DNA–protein complexes were immunoprecipitated with RNA polymerase II, c-jun, Jun B, Jun D and cAMP response element binding (CREB) antibodies or appropriate immunoglobulin G (IgG) isotype controls (Santa Cruz). PCR amplifications were carried out with appropriate primers spanning the human COL1A2 promoter and enhancer. These primers are human specific (see Supplementary Data for details).

A stable mouse cell line was created, cloned from a single NIH3T3 fibroblast transfected with a construct containing the human wild-type (WT) enhancer sequence fused to the proximal promoter in which the AP-1 site (at position –250 bp) has been abolished (21.1/18.8pLAC–ΔAP-1–250) and used in ChIP assays. Briefly, the (–21.1/18.8pLAC–ΔAP-1–250) construct was co-transfected in NIH3T3 cells with pCDNA3. After G418 selection, a stably transfected cell was picked and verified by β-galactosidase assay for highest expression and cloned.

Generation of transgenic mice

Transgenic mice were generated using standard method as described earlier (2). E15.5 embryos were harvested and stained for β-galactosidase as described (1,2).

RESULTS

Identification of a novel Smad-independent TGF-β response element in the *COL1A2* enhancer

Plasmid DNA constructs containing enhancer and/or promoter fragments driving reporter gene activity were used to determine the contribution of the human *COL1A2* far upstream enhancer compared with the proximal promoter to transgene expression. These plasmids were transfected into fibroblasts, treated with or without TGF-β, in the presence and absence of stimulatory Smad3/4 and inhibitory Smad 7.

In NIH3T3 cell line, construct 21.1/18.8pLAC containing a 2.3-kb region of the far upstream enhancer region fused to the proximal promoter resulted in a 6-fold increase of reporter gene activity in the presence of TGF-β compared with a 2-fold increase of the proximal promoter (Figure 1A). This result was also observed in explanted primary human dermal fibroblasts, the same construct (21.1/18.8pLAC) resulted in an ~3-fold increase of reporter gene activity in the presence of TGF-β compared with a 2-fold increase of the proximal promoter (PP) (data not shown). The subsequent transient transfection studies were carried out in NIH3T3 and/or primary human dermal fibroblasts interchangeably and with similar results.

The co-transfection of expression vectors for Smad3 (cmvSmad3) and Smad4 (cmvSmad4) with the proximal promoter construct (–378pLAC) increased the baseline response in both TGF-β-treated and -untreated cells, the over-expression of the Smad3 and Smad4 did not significantly alter the response of the enhancer construct (21.1/18.8pLAC) (Figure 1A).

Conversely, co-transfection of expression vector for the inhibitory Smad7 (cmvSmad7) with the proximal promoter construct resulted in the decrease of basal activity as well as the complete inhibition of the TGF-β response (Figure 1A). This is to be expected, as a Smad-dependent TbRE has been previously identified and extensively characterized within the proximal promoter (9,13).

However, what is of interest is the response of the construct containing both the enhancer and the promoter

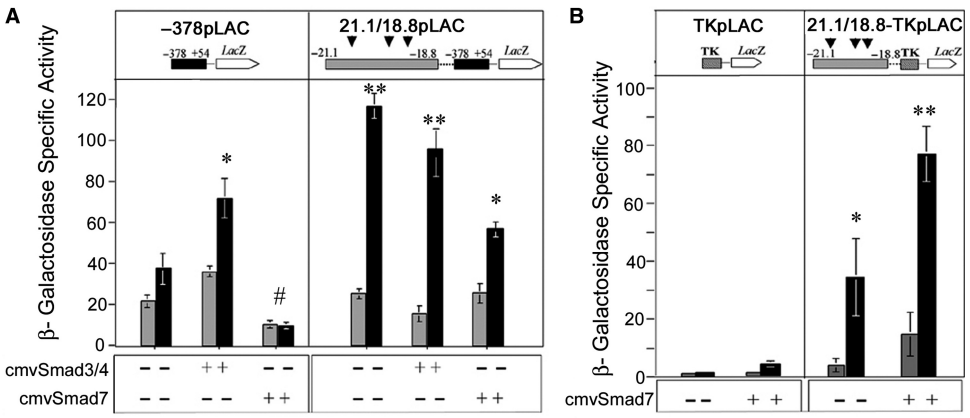

Figure 1. Smad-independent TGF-β activity associated with the COL1A2 far upstream enhancer region. (**A**) NIH 3T3 mouse fibroblasts were transiently transfected with β-galactosidase reporter gene constructs containing the COL1A2 proximal promoter alone or the COL1A2 enhancer fused to the proximal promoter. The fibroblasts were co-transfected with either a cmvSMAD7 expression vector to block Smad-dependent signalling, or an empty cmv vector as a control, and treated with (black bars) or without (grey bars) TGF-β. Cells were also tranfected with the same reporter gene constructs and co-transfected with or without cmvSmad3, cmvSmad4 or cmvSmad7 expression vectors. Reporter gene activity was measured. (**B**) NIH3T3 fibroblasts transiently transfected with β-galactosidase reporter gene constructs containing the TK proximal promoter alone or the COL1A2 enhancer fused to the TK proximal promoter. The fibroblasts were co-transfected with either a cmvSmad7 expression vector to block Smad-dependent signalling, or an empty cmv vector as a control in treated with (black bars) or without (grey bars) TGF-β. Data for (A) and (B) were obtained from independent experiments ($n = 6$, mean ± SEM. $^{*}P < 0.01$ and $^{**}P < 0.001$ significant increase with TGF-β treatment compared with untreated in the same transfection group. $^{\#}$Significant reduction compared with control in the presence of Smad 7).

when co-transfected with cmvSmad7. Although the level of reporter gene activity with TGF-β treatment decreased by 50%, it did not completely abolish the TGF-β response (Figure 1A). This suggested that part of the TGF-β response was Smad independent and associated with the 2.3 kb enhancer region.

To investigate this possibility further, new DNA constructs were prepared in which the proximal promoter region was replaced by a heterologous TK promoter. The TK promoter alone has no Smad-dependent TbRE and is not responsive to the canonical TGF-β signalling pathway. The TK promoter was fused to the 2.3-kb enhancer region and transfected into fibroblasts, it clearly responds to TGF-β treatment (Figure 1B). Furthermore, co-transfection with Smad7 did not affect the TGF-β-induced activation of the reporter gene in construct 21.1/18.8–TKpLAC, supporting the hypothesis of a Smad-independent TbRE in the far upstream enhancer region.

Further evidence supporting a Smad-independent mechanism is provided by transient transfections in Smad3 knockout (KO) fibroblasts completely lacking Smad3 compared with WT fibroblasts treated with and without TGF-β (Supplementary Data, Figure 1A). The promoter constructs 378pLAC and TKpLAC were transiently transfected in these cells and, as expected, did not respond to TGF-β. However, the constructs containing the enhancer region attached to the minimal promoter 21.1/18.8pLAC and 21.1/18.8-TKpLAC remained TGF-β responsive.

We also carried out a time course of TGF-β treatment on WT and Smad3 KO fibroblasts, RNA was isolated from cells and endogenous collagen type I northern blots were carried out using a COL1A2 specific probe. In Smad3 KO cells, a slow incremental increase of messenger RNA (mRNA) up to 40 h was observed which is more intense after TGF-β treatment (Supplementary Data, Figure 1B). In contrast, the WT cells produced more COL1A2 mRNA during the earlier time points peaking after 20 h and decreasing by 40 h (Supplementary Data, Figure 1B). The same pattern was observed in the presence of TGF-β, although the mRNA levels were overall higher. Furthermore, western analysis of Smad 3/4 in the presence of TGF-β did not change over 0–48 h (Supplementary Data, Figure 1C). However, in the same experiment, Jun B expression appeared after 6 h but no change in the expression levels of c-jun or JunD was observed (Supplementary Data, Figure 1D). These data add to the body of evidence that there is, in addition to a Smad-dependent component in the proximal promoter, a Smad-independent TGF-β response in the enhancer results in the transcriptional upregulation of *COL1A2* gene.

Mapping the novel TbRE in the enhancer and the role of upstream AP-1

In order to narrow down the region that contains the putative TbRE within the 2.3-kb enhancer, we generated smaller sub-constructs (F1, F2 and F3; Figure 2A) that spanned the footprint regions reported by Antoniv *et al.* (1). When these constructs were transfected in fibroblasts in the presence of expression vector cmvSmad7 (to inhibit the proximal promoter TbRE), only construct 20.17/19.94pLAC (Figure 2, F2) showed stimulation of reporter gene activity by TGF-β (Figure 2B). This result suggested that the 230-bp F2 sequence (Figure 2A) contained a TbRE and that this activity was not dependent on Smad signalling.

In silico surveying of putative transcription-factor-binding sites that may be present within the F2 sequence revealed the presence of two putative AP-1-binding sites (Figure 2A).

In order to investigate the role of the AP-1 sites further, we used a mutagenesis approach to generate DNA constructs that contained WT and AP-1 mutant sequences, both in the proximal promoter and within the enhancer. In the proximal promoter, a well-characterized AP-1 site was mutated and two constructs were created. The first mutant construct of the proximal promoter alone, where the AP-1 site at −250 bp was abolished (−378pLAC–δAP-1–250), was not active in transient transfections, either in the presence or absence of TGF-β. The same result was observed with the second mutant, which contained the WT enhancer fused to the mutant proximal promoter (21.1/18.8–δAP1–250) (Figure 3). In addition, four mutant constructs were created (21.1/18.8pLAC–δAP1–20.34, 21.1/18.8pLAC–δAP1–20.34/20.09, 21.1/18.8pLAC–δAP-1–20.03, 21.1/18.8pLAC–δAP-1–20.09/20.03) in which two point mutations were introduced in accordance with the AP-1 mutation analysis by Risse *et al.* (27). Three AP-1 sites found within the enhancer were used to generate constructs fused to a WT proximal promoter. Two of these sites are located within the F2 fragment (AP-1–20.03 and AP-1–20.09) (Figure 2), whilst the third, which is 215 bp upstream and outside the footprint region (AP-1–20.34), was used as a control.

These constructs were transfected in the presence of the cmvSmad7 expression vector and the presence/absence of TGF-β. The results revealed that mutation of the 5′AP-1 site located at –20.4 kb had no effect on either enhancer activity or the TGF-β response. However, the AP-1 site mutations within the footprinted sequence resulted in the blocking of the TGF-β response (Figure 3).

In order to examine which AP-1 family members may be involved in the regulation of the enhancer TbRE, we over-expressed c-fos, c-jun, Jun B and Jun D by themselves and in various combinations in NIH3T3 fibroblasts. The effect of the AP-1 family members was studied in fibroblasts transiently transfected with the F2 construct in the presence of cmvSmad7 and the presence or absence of TGF-β treatment (Supplementary Figure 2). Interestingly, Jun B or Jun B/c-fos stimulated the activity of the reporter gene, ∼4-fold and 3-fold, respectively, on TGF-β treatment. JunD/c-fos heterodimers also resulted in a 2-fold increase in reporter gene activity on TGF-β treatment, although over-expression of JunD alone had no effect compared with control (Supplementary Figure 2). Conversely, c-jun over-expression seemed to have an inhibitory effect potentially 'mopping-up' the endogenous levels of Jun B by forming heterodimers.

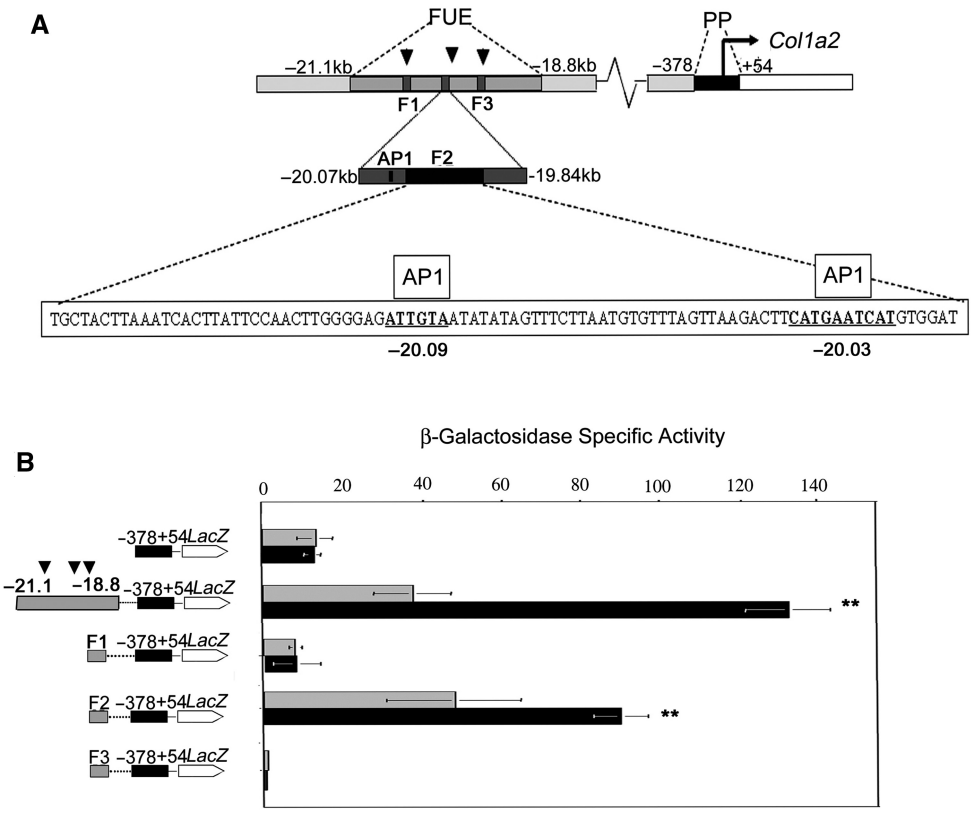

Figure 2. Mapping of the TbRE within the COL1A2 enhancer. (**A**) Schematic representation of the far upstream enhancer region (FUE) with arrows indicating known DNase hypersensitive sites and putative AP-1-binding sites and the proximal promoter (PP) of the human COL1A2 gene. (**B**) Reporter gene constructs containing regions of the COL1A2 far upstream enhancer which have been shown in fibroblasts to contain 'footprints' (F1, F2 and F3) of open chromatin were transfected in human primary dermal fibroblasts, co-transfected with cmvSmad7 expression vector to inhibit Smad signalling and treated with (black bars) or without (grey bars) TGF-β. ($n = 6$, mean ± SEM **indicates significant increase $P < 0.001$ with TGF-β treatment compared with untreated in the same transfection group).

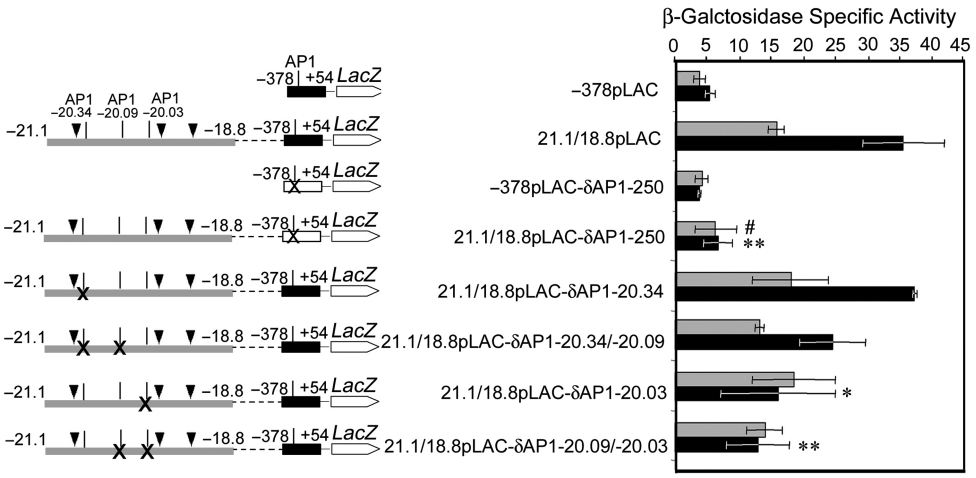

Figure 3. Mutation of key AP-1 sites in COL1A2 results in inhibition of transcriptional activity. Site-directed mutagenesis (x) was carried out on AP-1 consensus sites located either within the proximal promoter or the far upstream enhancer region. The mutated sequences transiently transfected with β-galactosidase reporter gene constructs containing either the enhancer F2 or the proximal promoter alone. Co-transfection with cmvSmad7 expression vector was used to block Smad-dependent signalling from proximal promoter in treated with (black bars) or without (grey bars) TGF-β ($n = 6$, mean ± SEM. $^{*}P < 0.01$ or $^{**}P < 0.001$ significant decrease with TGF-β treatment of mutated enhancer sequences compared with intact enhancer. $^{\#}$Indicates significant decrease ($P < 0.001$) in mutated proximal promoter in the presence of an enhancer compared with control, 21.1/18.8pLAC).

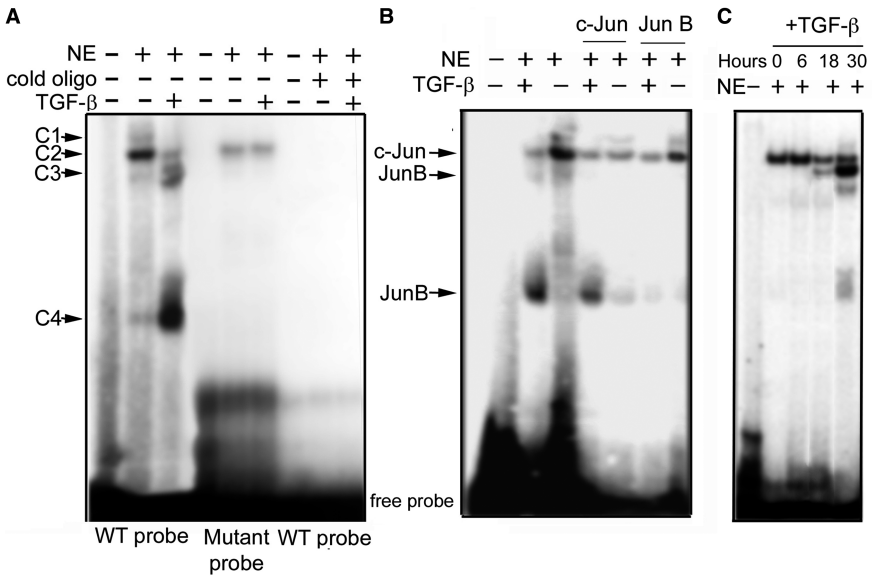

Figure 4. JunB displaces c-jun on the COL12 enhancer after TGF-β activation (**A**) EMSAs were carried out using a 24-bp labelled oligonucleotide probe spanning the AP-1 sites within the F2 COL1A2 enhancer region. Nuclear extracts (NE) from NIH3T3 fibroblasts treated or untreated with TGF-β were used in EMSA. Cold competitor oligonucleotides were also used to verify specificity of the DNA/protein complexes using the wild type probe. A mutant probe, in which AP-1 binding was abolished, was also used in EMSAs. (**B**) Super-shift assays were carried out using specific antibodies for either c-jun or Jun B in which NE were incubated with the antibodies prior to the addition of the probe. DNA/protein complexes (C1 to C4) were identified. (**C**) Time course of treatment with TGF-β showing Jun B binding with diminishing c-jun.

These experiments were repeated in Smad3-null fibroblasts and similar results were obtained (data not shown).

Having identified two putative AP-1 regulatory sites within the F2 fragment, their functional significance, in terms of TGF-β treatment, was further investigated using EMSA. Three overlapping double-stranded DNA (dsDNA) oligonucleotides (GS-30 bp, GS-50 bp and GS-24 bp) spanning the footprint region within 20.17/20.94 pLAC were synthesized and radiolabelled for the EMSA probes used with nuclear extracts from NIH3T3 cells treated with and without TGF-β (data not shown). Although DNA/protein complexes were observed with all three probes, the only ones that altered with TGF-β treatment were with the GS-24 bp probe (Figure 4). The GS-24-bp probe which contained the putative AP-1 site (–20.03 kb), used in binding reactions with untreated nuclear extracts revealed three DNA/protein complexes (C1, C2 and C3) (Figure 4A). In the presence of TGF-β-treated nuclear extracts, DNA/protein complex C1 was lost, C2 diminished, C3 increased and, a new complex, C4 appeared (Figure 4A and B). These complexes were specific as shown by the addition of the unlabelled competitor oligonucleotide. Furthermore, by mutating the AP-1 site within the GS-24 bp probe, all DNA/protein complexes were lost apart from C2 which diminished significantly (Figure 4A).

In addition, super-shift reactions using anti-c-Jun blocking antibody, complex C2 diminishes indicating that the shift contains c-jun (Figure 4B). Complexes C3 and C4 that are present only in the TGF-β-treated samples were diminished or abolished when anti-Jun B antibodies were used in the binding reactions, suggesting that these complexes contain Jun B, while complex C1 remains unaltered (Figure 4B). Isotype control antibodies [Sp1, epidermal growth factor receptor (EGFR)] were also used but had no affect on the binding in either the absence or presence of TGF-β (data not shown). A time course of up to 30 h with TGF-β shows a diminishing c-jun and a shift appearing for Jun B (Figure 4C). These data suggest that there is an alteration in the DNA/protein complex (s) that can form at position –20.03 kb after TGF-β treatment–from one that contains c-jun to one that contains Jun B.

The role of Jun B on *COL1A2* expression

Thus far, the data obtained suggested that AP-1 site at position –20.03 kb is involved in the transcriptional activation of the gene after TGF-β treatment and that Jun B is part of the DNA/protein complex that brings about this activation. Jun B is not highly expressed by untreated human skin fibroblast unlike c-jun and JunD, which are constitutively expressed. Furthermore, upon TGF-β activation, Jun B is upregulated and continues to be expressed for at least 48 h after treatment (Supplementary Figure 1D). Further experiments were carried out using siRNA for Jun B showing that transgene activity was also diminished in a dose–response manner, whilst control siRNA had no effect (Supplementary Figure 3A). The inhibition was evident in both TGF-β-treated and untreated cells, although the effect was greater in TGF-β-treated cells. Thus, depletion of Jun B resulted in a decrease of the activity of the F2 enhancer region in a dose–response manner in both TGF-β-treated and untreated cells. Interestingly, inhibition of Jun B not only blocked the TGF-β response but also the basal levels of enhancer activity, suggesting that Jun B is

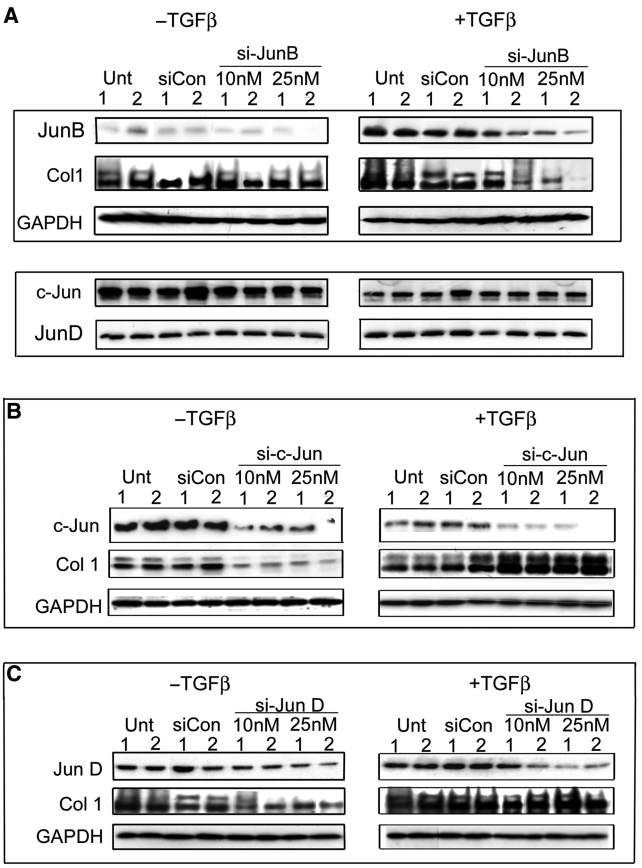

Figure 5. Effect of siRNA knockdown of AP-1 family on Collagen type I. Human primary dermal fibroblasts were treated with Jun B siRNA (10 nM and 25 nM) or a scrambled siRNA (25 nM) in the presence or absence of TGF-β. SDS–PAGE and western blot analysis of the protein levels of Jun B or Collagen type I (Col I) were assessed with specific antibodies (**A**). c-jun and Jun D levels do not change (A, lower panel) as with GAPDH used as a loading control. The fibroblasts were also treated with siRNA for c-jun (**B**) and Jun D (**C**).

involved in enhancer function. Similar results were also obtained with TK heterologous promoter (Supplementary Figure 3B).

We hypothesized that upon TGF-β activation, upregulation of Jun B will also lead to upregulation of collagen type I protein. Therefore, inhibition of Jun B expression using siRNA should result in inhibition of collagen type I protein. Indeed, specific inhibition of Jun B in a dose–response manner was achieved using siRNA on human dermal fibroblasts (Figure 5A). This siRNA treatment did not have an effect on the level of either c-jun or Jun D (Figure 5A, lower panel). Similarly, siRNA for c-Jun did reduce the collagen type I protein levels but not in the presence of TGF-β (Figure 5B), while siRNA for Jun D had very little effect on the collagen type I level in the presence of TGF-β (Figure 5C)

The role of Jun B on the transcriptional activity of the COL1A2 enhancer was further investigated *in vivo* in human dermal fibroblasts using ChIP assays (Figure 6A). The explanted fibroblasts were treated with and without TGF-β followed by fixation and fragmentation of the chromatin into fragments of around 500 bp. Immunoprecipitation was then carried out using RNA polymerase II antibody followed by purification of the DNA from the immunoprecipitated chromatin complexes and amplification using primers specific to the proximal promoter and the F2 fragment. RNA polymerase II is a multi-subunit DNA-binding enzyme responsible for transcriptional initiation by recruiting and co-ordinating the various components of the transcriptional complex, which interact with the gene promoter. As expected, RNA polymerase II co-precipitated with the proximal promoter DNA (PP) which contains the transcriptional start site in the cells which were both treated and untreated with TGF-β (Figure 6A). The F2 enhancer fragment also co-precipitated with RNA polymerase II immunoprecipitation. As RNA polymerase II does not bind directly on the enhancer, this result suggests that the F2 fragment of the enhancer is interacting directly with the proximal promoter in both TGF-β-treated and -untreated cells since c-jun precipitated with both the proximal promoter DNA (PP) and the F2 fragment (Figure 6A). However, there was a significant decrease of the F2 PCR product with TGF-β treatment, suggesting that less F2/c-jun complexes were available for amplification under these conditions. PP/c-jun complexes increased with TGF-β treatment.

Jun B did not precipitate with the F2 fragment and the level of PP PCR product was relatively low in the untreated cells; however, with TGF-β treatment both F2/Jun B and PP/Jun B complexes formed. These results are in agreement with the data that Jun B is not constitutively expressed in untreated skin fibroblasts, although it is increased with TGF-β treatment. The ChIP data clearly show that the TGF-β response of the enhancer is mediated through a decrease in F2/c-jun complexes and increase of F2/Jun B complexes interacting with the proximal promoter.

We have also used mouse cells cloned from a single NIH3T3 fibroblast stably transfected with a construct containing the human WT enhancer sequence fused to the proximal promoter in which the AP-1 site (at position –250 bp) has been abolished (21.1/18.8pLAC–δAP-1–250) in ChIP assays in order to investigate further if this site is involved in either the enhancer TGF-β response or in the recruitment of the enhancer to the promoter during this response. For comparison we have a stably transfected line of NIH3T3 fibroblasts containing the WT human enhancer and promoter sequences (21.1/18.8pLAC). After TGF-β treatment, ChIP assays were performed and the purified DNA was amplified using primers that can only PCR the human sequence, therefore allowing for the detection of the transfected DNA rather than the endogenous mouse DNA. ChIP assays were carried out using antibodies against RNA polymerase II, c-jun, Jun B, JunD and CREB (Figure 6B). In the WT cells, the results confirmed the data obtained in human dermal fibroblasts as previously shown in Figure 6A, that RNA polymerase II co-precipitated with the PP in both the absence and presence of TGF-β stimulation and co-precipitated the F2 fragment indicating that PP and enhancer are interacting. In WT cells c-jun occupies the PP and F2 enhancer region in the absence of TGF-β stimulation, whereas,

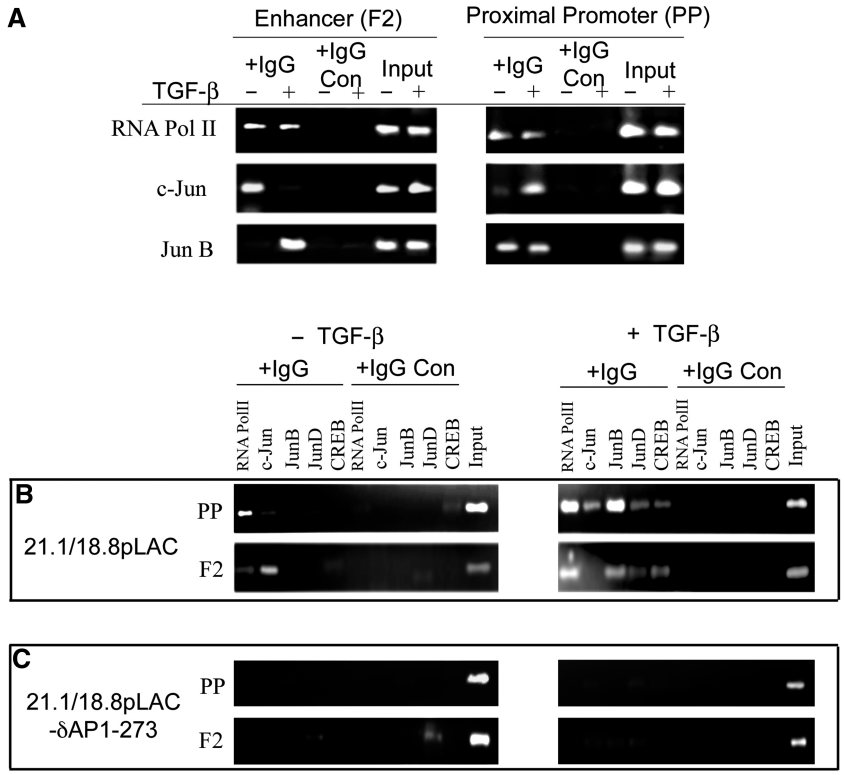

Figure 6. *In vivo* interactions of the COL1A2 enhancer and proximal promoter. (**A**) Primary human dermal fibroblasts treated or untreated with TGF-β were used in ChIP assays. PCR reactions were carried out using specific primers for the F2 region of the COL1A2 enhancer or the proximal promoter (PP). Ten percent of input was used as positive control. Antibodies for RNA polymerase II (RNA PolII), c-jun and JunB were used to immunoprecipitate the protein/DNA complexes. Appropriate isotype-specific controls were also performed. (**B**) ChIP assays were also carried out on NIH3T3 cell lines stably transfected with constructs containing either the wild-type human enhancer sequence fused to the proximal promoter (21.1/18.8pLAC). In (**C**), the wild-type human enhancer sequence fused to the proximal promoter in which the AP1 site at position –250 has been mutated (21.1/18.8pLAC–δ–AP-1–250). Antibodies for RNA PolII, c-jun, JunB, JunD and CREB were used to immunoprecipitate the protein/DNA complexes. Appropriate negative IgG controls were also performed.

upon TGF-ß treatment, Jun B binds more strongly to both the PP and F2 regions whilst the levels of F2/c-jun complexes decrease significantly (Figure 6B, left versus right panel). In addition, TGF-β stimulation also results in increased binding of Jun D and CREB on both the PP and F2 DNA (Figure 6B). However, in the stable cell line, where the AP1 site in the proximal promoter had been mutated (21.1/18.8pLAC–δAP-1–250), there appears to be little or no binding either of the AP-1 family members on the PP DNA or on the F2 enhancer region in absence of TGF-β, whereas TGF-β stimulation only slightly increases the level of binding (Figure 6C). Interestingly, the abolition of that AP-1 site in the PP results in the inability of RNA polymerase II to remain bound on the promoter, suggesting that AP-1 binding to this site has an essential role in the normal function of the transcriptional machinery (Figure 6C). Not surprisingly, F2/RNA polymerase II complexes are not observed indicating that the enhancer requires both an AP-1 site and RNA polymerase II containing complex before it can interact with the proximal promoter (Figure 6C).

To test the significance of this finding, transgenic mice were generated harbouring the intact enhancer and an AP-1 mutated 21.1/18.8pLAC–δAP-1–250 construct (Figure 7). The results showed a mutation in proximal promoter AP-1 leads to a complete lack of expression of the transgene in all the transgenic embryos obtained. The absence of even the minimal levels of expression observed in transgenic mice harbouring the proximal promoter alone, suggests that the AP-1 in the proximal promoter is necessary for the *in vivo* activity of the upstream enhancer as well as the proximal promoter of the *COL1A2* gene. A surprising result was also obtained when a mutation of the 21.1/18.8pLAC–δAP-1–20.03 construct was analysed in embryos showing a significant attenuation of the fibroblast transgene expression in all fibroblasts, including skin (Figure 7, lower right panel). Analysis of all embryos showed ~30% of pups are transgenics and 40% of those expressed the LacZ transgene. In each of the expressing lines, three embryos expressed the transgene in similar spacio-temporal fashion, indicating that the integration site does not affect the expression in significant manner.

DISCUSSION

TGF-β has a pleiotropic effect *in vivo*, exerted through a range of pathways, which involve different signal molecules depending on the target cell. Each of these pathways is initiated by the binding of transcription factors to target

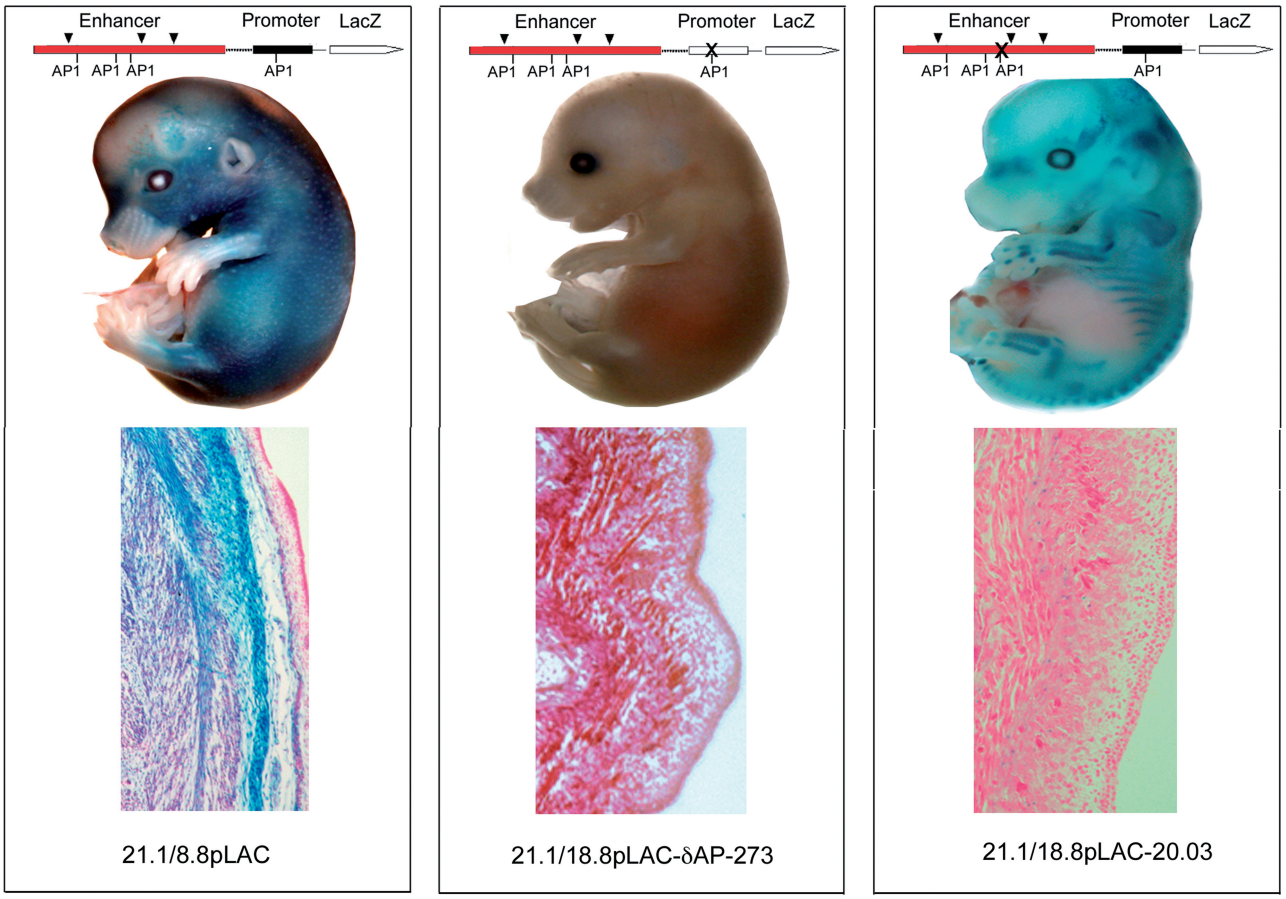

Figure 7. Mutation of AP-1 sites in proximal promoter and enhancer affect COL1A2 expression in transgenic mice. Transgenic mice were generated harbouring either the (21.1/18.8pLAC) shows expression in fascia of skin (left panel) and all collagen type I producing cells as previously shown. The 21.1/18.8pLAC–δ–AP-1–250 construct shows no expression in embryos and as illustrated in cross-section of skin (middle panel). However, 21.1/18.8pLAC–δ–AP-1–20.03 showed attenuated lacZ activity in fibroblasts of most organs and very little in skin sagital section (right panel).

genes. The Smad family constitutes the main transcription factors involved in TGF-β signalling, although others have been shown to bind to native DNA and associate with Smad proteins to induce activation/repression of target gene promoters (28,29). Some of the activating factors include Sp1, AP-1, Runx, Fox and bZip, to name but a few (30–32).

The central question in this study is: How does TGF-β activate the synthesis of collagen type I, especially in the light of the relatively recent discovery of the far upstream enhancer within the *COL1A2* gene which has been shown to play an important role in the full expression of collagen type I *in vivo* during development (8) in adult tissue repair (3) and in fibrotic mouse models (5,33).

At the molecular level, a series of experiments from several laboratories has shown that the *COL1A2* promoter harbouring a CAGACA consensus sequence in the proximal promoter between –378 and +52 bp, binds Smad3/Smad4 complexes, which mediate the TGF-β-induced *COL1A2* transcription in human skin fibroblasts. Furthermore, a cytokine response element (TbRE –313 bp to –250 bp) was coined to include a C/EBP site, three Sp1, CAGA and AP-1-binding sites which contribute to collagen gene regulation (9).

Within the TbRE, a *cis*-acting element containing a CAGA box was identified (between nucleotides −269 and −261) a sequence motif which binds Smad3/Smad4 complexes and is located directly 3′ of three Sp-1 transcription-factor-binding sites and 5′ of an AP-1-binding site. It was found that Smad 3/4 binding was not sufficient to drive TGF-β activation of the *COL1A2* gene. There is still some controversy as to the extent by which AP-1 versus Sp1 contribute to the TGF-β response of basal COL1A2. The three Sp1, between nucleotides –304 to −273 and TGF-β, were shown to be critical for COL1A2 transcription by increasing the affinity of an Sp-l-containing protein complex for its cognate DNA-binding site and complexing with Smad proteins (9–11). Chung *et al.* (13) showed that AP-1-binding site located 3′ of the TbREs plays a significant role in the basal activity of the promoter and TGF-β induction (14). Since these original findings, the discovery of the upstream enhancer and its role in coordinating the full response of *COL1A2* gene *in vivo* has

added a new complexity, which has been examined is this study.

The experiments outlined in this study show clearly that, in human adult fibroblasts and NIH3T3 cells, TGF-β engages the upstream enhancer not through Smad-binding sites but via complexes containing AP-1 family members. We verified this activity by using a heterologous minimal TK promoter, which has no TGF-β response element, by over-expression of Smad 7, an inhibitor of canonical TGF-β pathway, and in Smad3-null cells. We identified the sequence in the enhancer that is responsible for increased activity of *COL1A2* after TGF-β treatment and showed it to be located at around –20 kb away from the transcription start site, where two AP-1 binding sites were shown to be potentially involved. It is notable that this enhancer sequence spans a previously identified footprint (F2) in lung fibroblasts. Moreover, this region falls amongst two DNAse 1 hypersensitive sites (5 and 4) in chromatin described in our earlier publication and indicating the open transcriptional status of this region (1).

Expression vectors for Jun B and Jun D were found to significantly increase transgene levels of this enhancer sequence in the presence of TGF-β, while EMSA showed binding of Jun B to the identified elements and its knockdown significantly abolishes the effect of TGF-β on enhancer and reduces basal level of transcription. However, it is not fully understood why inhibition of Jun B in untreated cells reduces significantly the activity of the enhancer (Supplementary Figure 3) when expression levels of Jun B in untreated cells are low (Figure 5A). Clearly, the data suggest that there is a greater role for Jun B in the normal function of the enhancer.

AP-1 complexes form dimers of gene products of the Fos and Jun families, which have closely related recognition sites but different transcriptional activities and DNA-binding affinities. Moreover, modulation of AP-1/Jun expression by TGF-β is a cell-type-specific phenomenon, as TGF-β activates c-jun expression only in epithelial cells, whereas it induces Jun B in mesenchymal cells (34).

The ChIP data in this study clearly show a switch from c-jun to Jun B in the F2 sequence when TGF-β is added, while Jun B is always binding in the proximal promoter (Figure 6A) indicating perhaps that Jun B does relieve the repression of the c-jun exerted on *COL1A2*, as shown by Chung *et al*. The data from the ChIP assay experiments on fibroblast stable cell lines expressing the AP-1 mutant in the proximal promoter in the presence of the whole 2.3-kb enhancer are intriguing. The absence of the RNA polymerase II PCR product in those cells compared with WT construct indicate that the ability of the enhancer to engage with the proximal promoter is dependent on an AP-1 family member binding on the –250-bp site within the proximal promoter. The stability of this complex whether TGF-β induced or not, requires this interaction and appears to be mediated primarily by Jun B.

Based on the ChIP and *in vivo* evidence in this study, we propose that an AP-1 complex binding at –250 bp and enhancer (F2) plays an essential role in mediating the recruitment of the enhancer to the proximal promoter and stabilization of this interaction allows transcription of COL1A2

This suggestion is also based on other *in vivo* evidence of mutated key transcription-factor-binding sites (CBF, Sp1/Sp3) in the proximal promoter to assess the co-ordinate expression of enhancer/promoter during development (35). The results showed that tissue specificity and intensity of expression is altered suggesting that cooperation between the enhancer and basal promoter is required. Given that the mutation of AP-1 site in the proximal promoter in the presence of the enhancer diminishes the transcriptional read out of the *COL1A2* gene in transient expression abolishes the binding of the RNA polymerase II in ChIP assays and shows no expression in 40 transgenic embryos, when normally one would expect 40% expression, can only suggest that the functional AP-1 site in proximal promoter is required for the formation of an enhancesome, which recruits the upstream enhancer sequence to coordinate the expression of the collagen type I gene. TGF-β may be involved in this recruitment since there is a correlation of type I collagen gene expression during mouse embryogenesis with extracellular localization of TGF-β. What is notable is the expression in embryos which had two-point substitution in the AP-1 enhancer site. Almost complete attenuation of fibroblast expression not only in the skin but also in many internal organs compared to the enhancer suggesting perhaps that the AP-1 interaction along with other motifs may be critical for fibroblast-specific expression. Furthermore, we propose that the increase in transcriptional synergy when the enhancer is engaged, may be a reflection of an intrinsic DNA bend mediated by flanking sequences outside the core AP-1 recognition element site, which can strongly increase transcriptional activity as previously shown (36,37). Knowing the location of transcriptionally active DNAse I hypersensitive sites along the *COL1A2* promoter and given that repressors have been defined at –2.0 kb and +0.7 kb (1,38), we would speculate that the interaction between enhancer and proximal promoter in this gene favours DNA looping rather than scanning as a mode of enhancer–promoter cross-talk to activate the *COL1A2* gene. In summary, this study provides a paradigm for cytokine modulation of chromatin re-arrangement and provides yet another example of transcription-factor-dependent dynamic reconfiguration of long-range enhancer–promoter interactions. We propose a potential mechanism by which enhanced levels of *COL1A2* expression are achieved by TGF-β induction of Jun-B. A re-organization of the chromatin structure of *COL1A2* is followed by a displacement of c-jun by Jun B on a specific AP-1-binding site (−20.03 kb) within the far upstream enhancer region, resulting in the stabilization of enhancer/promoter interaction.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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