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# DIRECT DEMONSTRATION OF TRANSCRIPTIONAL ACTIVATION OF COLLAGEN GENE EXPRESSION IN SYSTEMIC SCLEROSIS FIBROBLASTS: INSENSITIVITY TO TGFß1 STIMULATION

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Lesional fibroblasts propagated from the skin of patients with scleroderma, when compared to normal fibroblasts, show increased synthesis of several collagens and increased levels of their corresponding mRNAs. Using constructs (COL1A2/CAT) containing the promoter for the alpha 2 (I) collagen gene in transient transfection assays with matched pairs of scleroderma and normal skin fibroblasts, we observed higher transcriptional activity of the COL1A2 gene in scleroderma fibroblasts and, in contrast to normal fibroblasts, no further expression was observed in the presence of TGFß1. Analysis of the expression of COL1A2 promoter deletion constructs indicates that the TGFß responsive element functional in normal fibroblasts and the sequence involved in intrinsic upregulation of COL1A2 gene expression in scleroderma fibroblasts are both located between bp -376 (Bgl II) and bp -108 (Sma I) sites. These data may indicate that intrinsic upregulation of extracellular matrix genes in scleroderma fibroblasts utilizes a TGFß dependent pathway. 

Scleroderma (systemic sclerosis; SSc) is a disease characterized by excessive deposition of connective tissue in the dermis, blood vessels and internal organs, in which lesional fibroblasts can be shown to produce increased quantities of extracellular matrix components on a per cell basis even after removal from the patient and propagation *in vitro* (1,2). Specifically, the increased synthesis of various collagen polypeptides is correlated with increased levels of corresponding mRNAs (3-5), at least in part due to increased transcription demonstrated in the fibroblasts of lesions of patients with localized scleroderma by run off assays for collagen α2(I) (6). In addition to elevated synthesis of extracellular matrix components, SSc fibroblasts exhibit altered growth regulation, responding preferentially to a combination of TGFß and PDGF AA due to a distinct upregulation of PDGFα receptors (7). Normal skin (NS) fibroblasts respond preferentially to bFGF (8).

Though the mechanism of activation of SSc fibroblasts is unknown, it is currently believed that immune cells (possibly antigen-driven autoimmune T-cells) participate in tissue injury and

Abbreviations used: SSc = systemic sclerosis (scleroderma); NS = normal skin; TGFß1=one of several isoforms of transforming growth factor beta; COL1A2 = The gene expressing the alpha two chain of type I collagen; CAT = chloramphenicol acetyltransferase.

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inflammatory reactions producing cytokines which activate fibroblasts to proliferate and to synthesize extracellular matrix (9). TGFß is particularly effective in inducing major quantitative pro-fibrotic effects both *in vivo* and *in vitro* (10-12). Several laboratories (13), including our own (14), have demonstrated that brief exposure of normal fibroblasts to TGFß induces a prolonged increase in collagen and fibronectin mRNA and protein expression, a phenotype similar to that of high collagen producing SSc fibroblasts. We felt it important to study extracellular matrix gene expression directly in fibroblasts from the generalized form of scleroderma (SSc). To further study the mechanism of this activation, we examined the effects of TGFß on transcription of the COL1A2 gene in SSc and NS fibroblasts. We observed that a COL1A2 promoter CAT construct was overexpressed in SSc fibroblasts, while, in contrast to NS fibroblasts, no further expression was stimulated by TGFß.

## MATERIALS AND METHODS

Cells: Fibroblasts were obtained by skin biopsy from affected areas (dorsal forearm) of patients with diffuse cutaneous SSc with < 2 yrs of skin thickening. Control fibroblasts were obtained by biopsy of healthy donors (within several days of SSc biopsy) who were matched with each SSc patient for age, sex, race and biopsy site, and were processed in parallel. Newborn foreskin fibroblasts were obtained from the delivery suites of affiliated hospitals. All biopsies were obtained with informed consent and institutional approval. Primary explant cultures were established in 25 $cm^2$ flasks in DMEM supplemented with 10% FCS, 2-mM glutamine, and 50 $\mu$g/ml gentamicin. Amphotericin (5 g/ml) was included for the first week only. Monolayer cultures were maintained at 37°C in 5% $CO_2$ in air. Fibroblasts between the third and fifth subpassages were used for experiments.

Plasmids used in transient transfections: COL1A1 CAT construct pWS-2.5/CAT and COL1A2 CAT construct pMS-3.5/CAT and Bgl II and Sma I deletion constructs based on this vector (15) were kindly provided by Dr. Francesco Ramirez. pSV2-CAT and pSV0-CAT were used as positive and negative controls, respectively. In some experiments pSV-ß-galactosidase control vector (Promega) was used as an additional internal control.

Transient transfection assay: Cells were maintained in DMEM-10% FCS and 10 $\mu$g/ml gentamicin. Fibroblasts were grown to 90% confluence in 10 cm dishes in DMEM-10% FCS. Monolayers were washed once with DMEM-10% FCS and cells were transfected with 20 $\mu$g of various plasmids by the calcium phosphate technique (16). After overnight incubation, medium was changed to DMEM 1% FCS. Where indicated, cells were stimulated with 1 ng/ml of TGFß1 (R&D Systems Inc.). After 40 hours of incubation cells were harvested in Tris HCl, pH 7.8 and fractured by three freeze-thaw-cycles. Total protein of the cytoplasmic extract was measured using the BioRad technique. Extracts were normalized for protein content and incubated with acetyl CoA and [$^{14}$C] chloramphenicol (Amersham Corp.) overnight at 37°C. After ethyl acetate extraction, the chloramphenicol and the acetylated products were separated by thin layer chromatography for 40 minutes in chloroform methanol (95:5), followed by autoradiography. Autoradiograms were scanned by laser densitometry and CAT activity for each autoradiogram was corrected to reflect the efficiency of transfection relative to the control plasmid pSV2-CAT. In some experiments, pSV-ß-galactosidase vector was used as an internal control to normalize for transfection efficiency. Both positive control plasmids produced similar results.

Statistical analysis: Differences between SSc and matched normal fibroblasts and between the same cell strain with and without TGFß treatment were analyzed using Student's T test for paired variables.

## RESULTS

In initial experiments we compared the transcriptional activities of 3.5 kb COL1A2 promoter segment (pMS-3.5/CAT) and 2.5 kb COL1A1 promoter segment (pWS-2.5/CAT) in transient

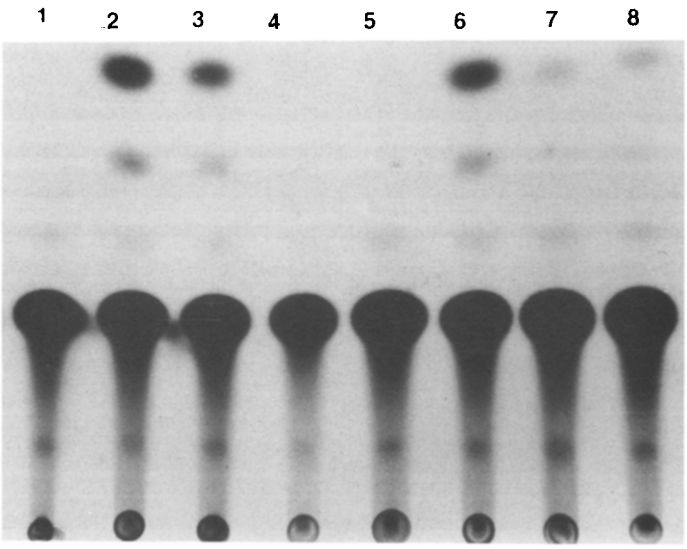


Figure 1. Activity of collagen CAT constructs in scleroderma (SSc) and normal (NS) fibroblasts. Transient transfection assays were performed as described in Methods. Three pairs of SSc (line 1-4) and NS (line 5-8) fibroblasts were tested in independent experiments; one representative experiment is shown. Lane 1 & 5, pSVO-CAT (negative control); Lane 2 & 6, pSV2-CAT (positive control); Lane 3 & 7, pMS-3.5/CAT; Lane 4 & 8, pWS-2.5/CAT. pMS-3.5/CAT is overexpressed in SSc fibroblasts (compare lanes 3 and 7).

transfection assays in SSc and control fibroblasts. The pMS-3.5/CAT vector exhibited higher activity in SSc fibroblasts than pWS-2.5/CAT (Figure 1); we therefore focused on the analysis of the COL1A2 promoter.

Transcriptional activity of the 3.5 kb COL1A2 promoter segment (pMS-3.5/CAT) was compared in seven pairs of SSc and NS fibroblasts and six newborn foreskin cell lines either untreated or stimulated with TGFß1. Matched pairs of SSc and NS fibroblasts were assayed together. As shown in Figure 2, basal COL1A2 promoter activity was higher for SSc fibroblasts than for NS fibroblasts ($1.41 \pm 0.27$ vs. $0.65 \pm 0.18$; $p = 0.0005$; $n=7$). When treated with TGFß1 normal fibroblasts increased COL1A2 expression from $0.65 \pm 0.18$ to $1.9 \pm 0.67$ ($p = 0.005$; $n=7$), while SSc fibroblasts did not increase COL1A2 expression ($1.41 \pm 0.27$ to $1.55 \pm 0.40$; $n=7$). Foreskin fibroblasts showed unstimulated levels and responded to stimulation by TGFß1 in a fashion similar to that of NS fibroblasts, increasing COL1A2 expression from $0.59 \pm 0.24$ to $1.20 \pm .30$ ($p = 0.0005$; $n=6$).

To identify TGFß responsive elements in normal fibroblasts and the cis-regulatory elements responsible for constitutive expression of COL1A2 in SSc fibroblasts, we used upstream-deletion constructs based on the vector pMS-3.5 CAT (15). These constructs were used in transient transfections with SSc and NS cells stimulated and unstimulated with TGFß1. As shown in Figure 3, both cis-regulatory elements are located between -376 (Bgl II) and -108 (Sma I). A TGFß responsive element in newborn foreskin fibroblasts is also located between Bgl II and Sma I (data not shown).

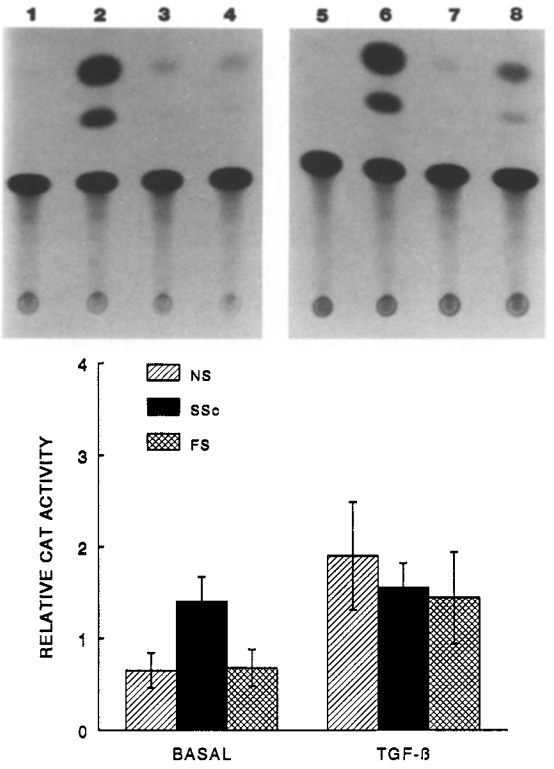


Figure 2. Activity of pMS 3.5/CAT construct in scleroderma (SSc), normal (NS) and newborn foreskin (FS) fibroblasts. Transient transfection, stimulation with TGFß1 and densitometry were performed as described in Methods. A representative experiment is shown on the top panel. Lane 1 & 5 pSVO/CAT; Lane 2 & 6 pSV2/CAT; Lane 3 & 7, pMS-3.5/CAT; Lane 4 & 8 pMS3.5/CAT & TGFß 1 ng/ml. Lane 1 to 4 SSc; Lane 5 to 8 NS. Diagram (bottom panel) represents COL1A2/CAT activity (mean ± SD) from seven pairs of scleroderma and normal fibroblasts and six foreskin fibroblasts cell lines, each experiment performed in duplicate dishes.

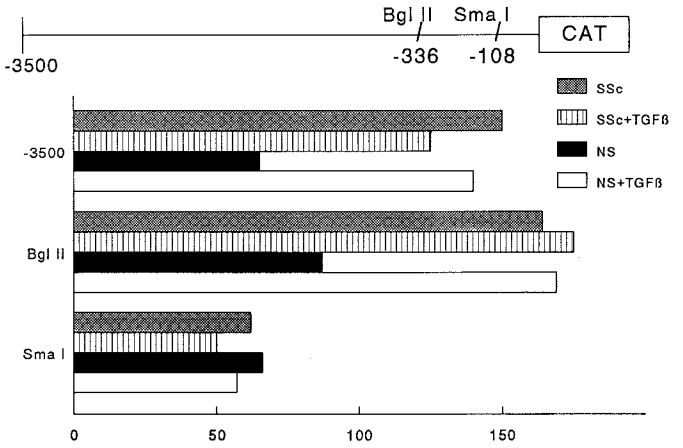


Figure 3. Deletion analysis of COL1A2 promoter in SSc and normal fibroblasts. At the top is a schematic representation of the COL1A2 promoter with the relative position of the Bgl II and Sma I restriction sites utilized for the generation of the CAT constructs. At the bottom, effect of deletions in the COL1A2 5' flanking sequences in SSc and NS fibroblasts stimulated or unstimulated with TGFß. Four pairs of SSc and NS cells were studied; a representative pair is depicted.

## DISCUSSION

In this study we demonstrated that the activity of the COL1A2 promoter is higher in SSc fibroblasts, when compared to closely matched NS fibroblasts. This finding, by an independent technique, confirms run off studies conducted using cells from children with localized scleroderma (6) and suggests that the over expression of collagen genes in scleroderma involves changes in transcriptional regulation. This is the first direct demonstration of increased transcriptional expression of an extracellular matrix gene in the cells of adult patients with systemic sclerosis (SSc).

In agreement with previous studies (17,18) we observed induction of COL1A2 transcription by TGFß in NS fibroblasts. However, TGFß had no effect on COL1A2 promoter activity in SSc fibroblasts. Thus, the COL1A2 promoter activity in SSc fibroblasts is comparable to the activity observed in TGFß stimulated NS fibroblasts. These data suggest that intrinsic upregulation of extracellular matrix genes in SSc fibroblasts could use a TGFß dependent pathway to upregulate the expression of matrix genes.

Analysis of COL1A2 promoter deletions indicates that TGFß responsive element in NS fibroblasts and the sequence involved in intrinsic upregulation of extracellular matrix genes in SSc fibroblasts are located between bp -376 (Bgl II) and bp -108 (Sma I). Previous experiments using mouse COL1A2 (17) or rat COL1A1 (19) promoters identified DNA cis-regulatory elements involved in mediating TGFß stimulation. These elements are similar to a consensus sequence for NF-1 (nuclear factor-1) binding protein. NF-1 consensus sequences compete for binding at these sites. An inverted NF-1 like sequence (CCGCCCTTTCCAAG) is present between bp -298 and bp -311 in the human COL1A2 promoter and shows only a two nucleotide difference when compared to TGFß responsive element in mouse COL1A2 promoter (TCGCCCTTGCCAAG). This suggests a similar mechanism for TGFß stimulation of COL1A2 transcription in mouse and human exists. However, further studies are required to confirm this possibility and to determine whether the same cis-regulatory element is also responsible for upregulating COL1A2 transcription in SSc fibroblasts. The NF-1 responsive element is also present in the c-myc promoter (20) and elevated c-myc mRNA levels are observed in SSc fibroblasts (21).

In previous studies, we demonstrated that TGFß differentially modulates proliferative responses to other growth factors in normal adult and newborn foreskin fibroblasts (7,8), while, as shown in this study, effects of TGFß on collagen transcription are similar in the two cell types. These observations suggest that in human fibroblasts separate pathways may be involved in TGFß stimulation of matrix genes and TGFß effects on proliferation. SSc fibroblasts differ from the other two cell types in both proliferation and matrix synthesis. It is now feasible to test whether different levels or activities of transacting factors involved in regulation of matrix gene expression and growth may be responsible for the SSc phenotype.

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