Sp1 Is Required for the Early Response of α2(I) Collagen to Transforming Growth Factor-β1*

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It is currently debated whether AP1 or Sp1 is the factor that mediates transforming growth factor β1 (TGF-β) stimulation of the human α2(I) collagen (COL1A2) gene by binding to an upstream promoter element (TbRE). The present study was designed to resolve this controversy by correlating expression of COL1A2, AP1, and Sp1 in the same cell line and under different experimental conditions. The results strongly indicate that Sp1 is required for the immediate early response of COL1A2 to TGF-β and AP1 is not. The Sp1 inhibitor mithramycin blocked stimulation of α2(I) collagen mRNA accumulation by TGF-β, whereas the AP1 inhibitor curcumin had no effect. Furthermore, antibodies against Jun-B and c-Jun failed to identify immunologically related proteins in the TbRE-bound complex, irrespective of whether they were purified from untreated or TGF-β-treated cells. AP1 did bind to the TbRE probe in vitro, but only in the absence of the upstream Sp1 recognition sequence. Based on this finding and DNA transfection results, we conclude that the AP1 sequence of the TbRE represents a cryptic site used under experimental conditions that either eliminate the more favorable Sp1 binding site or force the balance toward the less probable. Finally, a combination of cell transfections and DNA-binding assays excluded that COL1A2 transactivation involves the retinoblastoma gene product (pRb), an activator of Sp1, the pRb-related protein p107, an inhibitor of Sp1, or the Sp1-related repressor, Sp3.

Type I collagen is the major structural component of connective tissue and consequently one of the key contributors to embryonic development and growth and to tissue repair and homeostasis (1, 2). Deregulated type I collagen synthesis is invariably associated with disorders characterized by excessive matrix deposition or abnormal matrix degradation (3, 4). Hence, transcription of the genes coding for the α1 and α2 subunits of type I collagen is critical to many physiological activities and pathological processes. During the past few years, substantial effort has been devoted toward understanding the molecular mechanisms that control transcription of the type I collagen genes (5, 6). Some of the studies have examined tissue specific expression of the murine α1(I) and α2(I) collagens in transgenic animals; others have focused on the response of the human genes to cytokines. Altogether, the analyses suggest that the regulatory sequences of these coordinately expressed genes are organized somewhat differently. On the one hand, transcription of the murine α1(I) collagen gene in distinct mesenchyme lineages appears to be under the control of separate cis-acting elements scattered within 3.5 kilobase pairs of upstream sequence (7, 8). On the other hand, spatio-temporal specificity of the mouse α2(I) collagen (Col1a2) gene seems to be confined to the 350-bp proximal promoter and cis-acting elements with redundant GC-rich binding sites for nuclear proteins (9–12).

One of the cis-acting elements of the mouse 350-bp promoter has been shown to participate in α2(I) collagen expression in dermal and tendon fibroblasts, and to mediate the transcriptional response of the gene to transforming growth factor-β1 (TGF-β) (9, 10, 13). Multiple copies of the −315 to −284 sequence of the Col1a2 gene were sufficient to drive transcription from the basal 40-bp promoter in transfected fibroblasts and only in the tail and skin of transgenic mice (9, 10). The finding was in line with prior results, which had correlated mutations introduced within the −315 to −284 sequence with a 10-fold loss of promoter activity (14). The same region had also been found to mediate the stimulatory effect of TGF-β through a CTF/NF-I sequence by an as yet undetermined mechanism (13). Subsequent work could not confirm the contribution of the CTF/NF-I binding site to TGF-β responsiveness and instead implicated AP1 without, however, documenting where the factor binds or what the transactivating mechanism might be (15). A more recent report has made a correlative argument to link CTF with the antagonistic signals of TGF-β and tumor necrosis factor-α (TNF-α) on Col1a2 gene expression (16). Work on the human α2(I) collagen (COL2A1) gene has reiterated the importance of the corresponding −330 to −283 promoter region in controlling fibroblast specificity and in mediating TGF-β responsiveness (17–19). It has also raised the same controversy as the mouse studies did. Whereas there is general agreement excluding the participation of CTF/NF-I in the regulation of the human gene, different reports have involved distinct cis-acting elements and trans-acting factors in constitutive and TGF-β-stimulated transcription of COL2A2 (18–20).

One set of studies has shown that the TGF-β and TNF-α signaling pathways converge on the same transcriptional

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1 The abbreviations used are: bp, base pair(s); AP, activating protein; CAT, chloramphenicol acetyltransferase; CTF/NF-I, CCAAT box transcription factor/nuclear factor I; EMSA, electrophoretic mobility shift assay; PMA, phorbol 12-myristate 13-acetate; pRb, the product of the retinoblastoma (Rb) gene; Sp1, specificity protein 1; TbRC, transcriptional complex bound to the TbRE; TbRE, TGF-β-responsive element; TGF-β, transforming growth factor-β1; TNF-α, tumor necrosis factor-α.
plex bound to the −330 to −255 region of the COL1A2 promoter (18, 21). The sequence consists of two nearly juxtaposed footprint patterns (Boxes A and B, see Fig. 1), the most distal of which was further subdivided into two (Boxes 5A and 3A) by the following criteria. Deletion of Box 5A increased promoter activity, whereas nucleotide substitutions in Box 3A nearly abrogated it; additionally, distinct nuclear proteins bound Boxes 5A and 3A independently of each other. Although Box 5A contains the counterpart of the murine CTF/NF-I binding site (Fig. 1), re-combinant NF-I failed to recognize the human sequence; furthermore, deletion of Box 5A had virtually no effect on the ability of TGF-β to stimulate the collagen promoter (18). TGF-β responsiveness was instead linked to Boxes 3A and B (ThrRE) and the cognate nuclear protein complex (ThrRC). Additional in vitro evidence indicated that one of the ThrRC components is the ubiquitous activator Sp1 (18). Most importantly, it was noted that TGF-β stimulation of COL1A2 gene expression translated into increased intensity of the ThrRC in the electrophoretic mobility shift assay (EMSA) (18). A subsequent study suggested that tyrosine dephosphorylation may be an obligatory step in ThrRC transactivation (22). These observations led to the formulation of the following hypothetical mechanism for TGF-β induction of COL1A2 transcription. TGF-β transactivates the ThrRC by modifying an Sp1 co-factor through a tyrosine dephosphorylation-dependent nuclear step. In turn, the post-translational modification increases either the recruitment of ThrRC to the cognate binding site or the affinity of the prebound complex. It was also hypothesized that the antago-

nistic signal of TNF-α affects both ThrRC and CIR, the alleged repressor that binds to Box 5A (21); the conclusion was based on the observation that overexpression of c-jun blocked cyto-
kine stimulation of the co-transfected collagen promoter (19). The above findings have been subsequently challenged by another study which located the TGF-β-responsive element to an AP1 recognition sequence overlapping the 3′ end of Box B (Fig. 1) (19). The study has also suggested that transcriptional stimulation of COL1A2 by TGF-β is due to a switch in Fos partners, from c-Jun to Jun-B, resulting from TGF-β induction of jun-B gene expression (19, 23). The idea has received further support from the independent finding that overexpression of jun-B induced transcription of the mouse Col1a2 gene, and antisense jun-B RNA attenuated its stimulation by TGF-β (15). It should be noted that another study similarly implicated TGF-β induction of jun-B expression in the down-regulation of the matrix metalloprotease-1 gene in cultured dermal fibro-

blasts (24). Hence, TGF-β may modulate matrix remodeling by changing the composition of a transcriptional complex that regulates expression of collagen and collagenase genes in opposite ways (19, 23, 24).

It was the principle objective of the present study to resolve the above controversy by elucidating whether the factor involved in TGF-β stimulation of COL1A2 transcription is Sp1 or AP1. To this end, we investigated how different experimental conditions affect expression of the candidate regulators and the target gene in the same fibroblast cell line. Within the limita-

tion of this in vitro experimental model, the results excluded AP1 participation in the early response of COL1A2 to TGF-β and, indirectly, confirmed Sp1 involvement in this process. They also excluded roles for availability of active Sp1 and release of Sp1 in ThrRC transactivation.

MATERIALS AND METHODS

Reagents—TGF-β1 and Verrasina-derived protein-tyrosine phosphatase were purchased from Boehringer Mannheim. Curcumin, mithra-
mycin A, cycloheximide and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. Oligonucleotides containing consensus recog-
nition sequences for Sp1 and GATA factors and for wild type and mutant AP1, as well as antibodies against Sp1 (PEP-2), c-Jun (D and N), Jun-B (N-17), pRb (C-15), and p107 (SD9) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Additional antibodies against Sp1 and Sp3 were generously provided by Dr. J. Horowitz (Duke University Medical Center, Durham, NC). The Rb expression vector, pHBl, and the parental plasmid, pBj1874, were a kind gift of Dr. R. Weinberg (Whitehead Institute for Biomedical Research, Cam-
bridge, MA); details about the construction of the COL1A2/CAT chimeric plasmids have been published (17, 18). cDNA probes for c-jun and jun-B were kindly provided by Dr. T. Curran (Roche Institute of Molec-

ular Biology, Nutley, NJ), whereas the Sp1 cDNA was a generous gift of Dr. J. T. Kadonaga (University of California, San Diego, La Jolla, CA). The DNA and human glyceraldehyde-3-phosphate dehydrogenase was purchased from American Type Culture Collection (Rockville, MD), and the COL1A2 cDNA has been described previously (26).

Nuclear Blot Hybridizations and Run-on Transcription Assays—Confluent CF-37 fibroblasts were placed in low serum 18 h before TGF-β addition. Likewise, cells were preincubated with curcumin (20 μM) for 30 min or with mithramycin (100 nM) for 18 h prior to TGF-β administration. In some experiments, cycloheximide and PMA were added at final concentrations of 0.05 mg/ml and 60 ng/ml, respectively. Viability of the cells was estimated using the trypan blue (0.4% in PBS) exclusion test; in all cases, cellular viability was more than 90%. Total RNA was extracted 4 h after the addition of TGF-β and processed for Northern blot hybridizations according to standard protocols (25, 27). Likewise, a standard protocol was used to determine rates of COL1A2 transcription in control and mithramycin-treated cells using glyceral-
dehyde-3-phosphate dehydrogenase and pBR322 DNA as controls (28). Quantitative data were obtained using the computer program NIH-

image software after scanning the autoradiographs with Adobe Photo-

shop (Adobe Systems Inc., Mountain View, CA).

Preparation of Nuclear Extracts—Nuclear extracts were prepared from control and TGF-β-treated fibroblasts, alone or in combination with cycloheximide. Confluent cultured for 18 h in low serum prior to the addition of either TGF-β or PMA. Cells were preincubated with curcumin as described above. Cells were har-

vested 3 h after treatment and processed to prepare nuclear extracts according to the published protocol (29).

DNA Binding Assays—Nuclear extracts were used in the EMSA with COL1A2 oligonucleotides spanning from −313 to −183 (ThrRE) and from −271 to −235 (Box B). Although longer than the ThrRE and Box B, the EMSA probes are nevertheless identified by the names of these cis-acting elements. Relevant to the present study, the ThrRE includes the Sp1 and AP1 recognition sequences and Box B encompasses only the AP1 binding site (Fig. 1). Oligonucleotides were end-labeled with [32P]dCTP using the Klenow fragment of DNA polymerase or with [γ-32P]ATP and T4 polynucleotide kinase (27). EMSAs were performed as described previously (18, 29). For mithramycin treatment, DNA probes were preincubated for 1 h at 4 °C in the presence of the drug (100 nm final concentration) before being added to nuclear extracts. In the competition experiments, unlabeled oligonucleotides were always added at 100-fold molar excess. The ThrRC pattern shown in the present and past studies (18, 21, 22) was reproducibly obtained only with hypotaurine cells, and not plasmid according to the protocols of Truter et al. (29) or Andrews and Fuller (30). In the antibody interference assays, 2 μg of antibodies were preincubated with 5 μg of nuclear extracts for 1 h at 4 °C before adding labeled probes. For the immunodepletion experiments, 20 μg of antibodies were conjugated to protein G-agarose beads in PBS and, after washing, they were incubated for 3 h at 4 °C with 5 μg of nuclear proteins. Following centrifu-
Inagaki et al. (18) originally proposed that TGF-β stimulates COL1A2 transcription by modifying the transactivating potential of an Sp1-containing complex (TbRC) that binds in vitro to a 130-bp probe (−313 to −183) inclusive of the TbRE (Fig. 1). In contrast, Chung et al. (19) have recently argued that TGF-β stimulation is mediated by the cytokine-induced change in the composition of the AP1 complex bound to the −265/−241 sequence from Fos/c-Jun to Fos/Jun-B (Fig. 1). We have revisited this important issue using a strategy that took into account the biological implications of both models. Specifically, we have compared the effects of various experimental conditions on the expression of the endogenous COL1A2 gene and the candidate regulators Sp1 and AP1 on the in vitro binding of the purified nuclear proteins.

**TGF-β Stimulates Jun-B and COL1A2 Expression Independently of Protein Synthesis**—It is well established that TGF-β stimulates collagen production by acting in part at the transcriptional level (32–34). It is also widely appreciated that a variety of factors, including culture conditions and the target cell, play a role in determining the precise mode of action of the cytokine and thus, the nature and extent of the response (35, 36). In view of these considerations, we first established how TGF-β modulates expression of the genes coding for α2(I) collagen, Sp1, and Jun-B in our experimental system, primary fibroblasts (CF-37) derived from human fetal skin (17).

The AP1 model predicts synthesis of the Jun-B protein as an intermediate step in TGF-β stimulation of COL1A2 (19). This prediction is consistent with the reported induction of jun-B mRNA accumulation within 1 h of TGF-β administration in several cell culture systems, including lung epithelial cells, foreskin fibroblasts and epidermal keratinocytes (34, 35). A rapid and substantial increase of jun-B mRNA after TGF-β treatment was also seen in our dermal fibroblasts cultures; like other cell culture systems, the increase was unaffected by co-administration of cycloheximide at a concentration known to nearly abolish protein synthesis in fibroblasts (37) (Fig. 2A). A more delayed stimulation was seen in duplicate Northern blots

**Mithramycin Blocks TGF-β Stimulation of COL1A2**—Curcumin and mithramycin are inhibitors of AP1 activation and Sp1 binding, respectively (36, 38). These agents were therefore used to compare the consequences of blocking the activity of each nuclear factor on COL1A2 gene regulation. Two different batches of nuclear extracts were analyzed by EMSA using high affinity recognition sequences for AP1 and Sp1. The first batch of extracts was prepared from CF-37 cells cultured with and without TGF-β and with and without curcumin. An aliquot from each fibroblast culture was processed in parallel for Northern analysis. Since mithramycin inhibits DNA binding by modifying GC-rich sites, the AP1 and Sp1 oligonucleotides were incubated in vitro with the drug prior to incubation with nuclear proteins purified from cells cultured for 6 h with (+) and without (−) TGF-β and probed against the anti-Sp1 antibody. Positions of molecular mass standards are marked.

**Results**

Western Blot Analysis—Nuclear extracts (15 μg for Sp1 detection and 20 μg for Rb detection) were electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel (−7.5% polyacrylamide) and transferred onto nitrocellulose filters as described by Towbin et al. (31). Filters were probed with antibodies (1:4000 dilution), followed by incubation for 1 h with horseradish peroxidase-conjugated rabbit anti-goat IgG diluted 1:3000. Sp1 and pRb were detected using an enhanced chemiluminescent system (ECL, Amersham Corporation) according to the manufacturer’s recommendations.

**FIG. 1. Nucleotide composition of the TbRE region.** The Sp1 and AP1 binding sites are underlined, whereas the sequences of Boxes 5A, 3A, and B are bracketed. The putative CTF/NF-I binding site of Box 5A is highlighted by the dotted line. The mutant version of the AP1 site used in this experiment is shown in lowercase letters. (A) Nucleotide composition of the TbRE region. (B) Nucleotide composition of the Sp1 binding site. The putative CTF/NF-I binding site of Box 5A is highlighted by the dotted line. The mutant version of the Sp1 site used in this experiment is shown in lowercase letters.

**FIG. 2. TGF-β modulation of fibroblast gene expression.** Panel A, Northern analysis of RNA purified from untreated cells and from cells exposed to TGF-β for the indicated times (in hours), with or without addition of cycloheximide (cyhx). The identity of each probe is shown on the right of the autoradiograms GAPD, glyceraldehyde-3-phosphate dehydrogenase. Panel B, immunoblot analysis of nuclear proteins purified from cells cultured for 6 h with (+) and without (−) TGF-β and probed against the anti-Sp1 antibody. Positions of molecular mass standards are marked.

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EMSAs confirmed the specificity of the two drugs in the selective elimination of either Sp1 or AP1 binding (Fig. 3A). Northern hybridizations established a causal association between inhibition of Sp1 binding and loss of COL1A2 stimulation by TGF-β (Fig. 3B). More importantly, they documented that curcumin inhibition of AP1 activity affects neither COL1A2 basal expression nor its stimulation by TGF-β (Fig. 3B). Consistent with the reported role of Sp1 in maintaining constitutive COL1A2 expression (39), α2(I) collagen mRNA levels decreased about 30% in uninduced cells after mithramycin treatment (Fig. 3B). Nuclear run-on experiments confirmed
that mithramycin acts mostly by repressing COL1A2 transcription (Fig. 3C). The results therefore strongly implicated Sp1 in the \textit{in vivo} modulation of COL1A2 gene expression by TGF-\(\beta\), while concomitantly excluding AP1 from this process. 

\textit{Sp1 Binding to the TbRE Is Favored over AP1}—The hypothesis of a TGF-\(\beta\)-induced switch in the composition of the AP1 complex is chiefly based on the observation that Jun-B and not c-Jun antisera affect \textit{in vitro} binding to the \(-265/-241\) sequence of nuclear proteins from stimulated cells (19). Here, the same test was repeated using CF-37 fibroblasts and probes that include either the AP1 binding site of Box B (probe \(-265/-241\)) or the AP1 and Sp1 recognition sequences of the TbRE (probe \(-313/-183\)). The former is the same probe used in the study that has led to the formulation of the AP1 model (19). In agreement with the mRNA expression levels (Fig. 2), the amount of Jun-B bound to the \(-265/-241\) probe increased following TGF-\(\beta\) treatment (Fig. 4A). The c-Jun antisera interfered with AP1 binding almost as effectively in control as in TGF-\(\beta\)-treated samples (Fig. 4A). This last result is in complete disagreement with the finding of Chung et al. (19), who reported the absence of immunologically identifiable c-Jun in the AP1 complex of TGF-\(\beta\)-treated cells. Unfortunately, this discrepancy cannot be resolved since the authors of that report did not examine the composition of AP1 in uninduced cells (13). The results of the immunointerference assays using the TbRE probe further questioned the validity of the AP1 model.

Despite including the \(-265/-241\) sequence and thus the AP1 site, the TbRE probe bound a complex without immunologically identifiable Jun subunits irrespective of whether the extracts were derived from untreated or TGF-\(\beta\)-treated cells (Fig. 4B). Consistent with previous findings (18), the Sp1 antisera eliminated binding of the top two TbRC bands (compare \textit{the first four lanes} with \textit{the last four lanes} of Fig. 4B). The results therefore indicated that AP1 can not bind to the cognate site in the presence of Sp1. We also noted that cycloheximide did not block the TGF-\(\beta\) induced TbRC binding (Fig. 4B). This observation further strengthened the positive correlation we had already established between the responses to TGF-\(\beta\) of the TbRC and the endogenous COL1A2 gene. A positive correlation

was also established in mithramycin-treated cells between inhibition of endogenous COL1A2 gene expression and loss of binding to the \(-313/-183\) probe (Figs. 3B and 4C). The correlation was substantiated by the finding that curcumin affected neither accumulation of \(\alpha 2(1)\) collagen mRNA nor binding to the \(-313/-183\) probe (Figs. 3B and 4C). We will discuss later the possible reasons behind the elimination of the whole TbRC by the Sp1 antisera in the mithramycin-treated sample.

Since AP1 could bind the site overlapping Box B under particular \textit{in vitro} conditions, we thought the same may also happen in transient transfection experiments. To this end, we compared the response of the transfected \(-378\)COL1A2/CAT plasmid and of the endogenous COL1A2 gene in CF-37 cultures treated with PMA, an AP1 inducer and a collagen inhibitor. The comparison documented the paradoxical result of PMA up-regulation of the former and down-regulation of the latter (Fig. 5). As predicted by the above experiments, curcumin blocked PMA-induced down-regulation of the endogenous COL1A2 gene (Fig. 5B). On the other hand, mutation of the AP1 site overlapping Box B did not interfere with the excision of the COL1A2/CAT plasmid and of the endogenous COL1A2 gene in CF-37 cultures treated with PMA, an AP1 inducer and a collagen inhibitor. The comparison documented the paradoxical result of PMA up-regulation of the former and down-regulation of the latter (Fig. 5). As predicted by the above experiments, curcumin inhibited PMA-induced down-regulation of the endogenous COL1A2 gene (Fig. 5B). On the other hand, mutation of the AP1 site overlapping Box B did not interfere with the excision of the COL1A2/CAT plasmid and of the endogenous COL1A2 gene in CF-37 cultures treated with PMA, an AP1 inducer and a collagen inhibitor. The comparison documented the paradoxical result of PMA up-regulation of the former and down-regulation of the latter (Fig. 5). As predicted by the above experiments, curcumin inhibited PMA-induced down-regulation of the endogenous COL1A2 gene (Fig. 5B).

\textit{Sp1 Is Not the Limiting Factor in COL1A2 Transactivation}—The evidence described so far was overwhelmingly in support of Sp1 involvement for the early response of COL1A2 to TGF-\(\beta\). Although a hypothetical mechanism has been proposed to explain how the Sp1-containing complex induces COL1A2 transcription, the details of it are far from being clear. For example, TGF-\(\beta\) could facilitate TbRC formation indirectly, by releasing an inhibitor from one of its components or directly, by changing its composition. We explored these possibilities as they apply to Sp1, the only known component of the TbRC.

It has been shown that TGF-\(\beta\) dephosphorylates the retino-
blasta protein, pRb (40); it has also been reported that pRb stimulates Sp1 transactivation either by liberating it from a specific inhibitor or by interacting directly with Sp1 (41–43). It was therefore formally possible that in our system TGF-β may increase the pool of active Sp1 molecules via pRb dephosphorylation. We investigated this possibility by examining the consequences of Rb overexpression on the endogenous COL1A2 gene and the transfected −378 promoter. To this end, CF-37 fibroblasts were co-transfected with the −378COL1A2/CAT construct and the pHbR or the pBJ1874 plasmid (22). Transfected cultures were processed in parallel for protein and RNA analyses and CAT assays. Western analysis documented the production of larger amounts of pRb in the pHRb-transfected compared with the pBJ1874-transfected cells and showed that most of it was dephosphorylated (Fig. 6A). Unlike the slight increase of c-Jun, Northern analysis revealed that α2(I) collagen mRNA levels remained virtually unchanged in Rb-overexpressing fibroblasts compared with control cells (Fig. 6B). Likewise, the CAT assays illustrated that Rb overexpression had no significant impact on the relative stimulation of the −378 promoter by TGF-β (Fig. 6C). Therefore, Rb seems not to play a role in TGF-β stimulation of COL1A2 transcription.

A candidate for Sp1 inhibition is the pRb-related protein p107 (44). To assess the potential involvement of p107, as well as to independently confirm the above conclusion, nuclear extracts from untreated cells were depleted of p107 or Rb using specific antibodies coupled to agarose beads. As a positive control, nuclear extracts were immunodepleted with Sp1 antisera. The unbound material was then incubated with the TGF-β, with or without prior treatment with a tyrosine phosphatase enzyme. Finally, the resulting EMSA patterns were compared with those of nuclear extracts which had not been subjected to immunodepletion (Fig. 7A). In designing these experiments, we assumed that the change of EMSA pattern observed with nuclear extracts from TGF-β-treated cells was functionally equivalent to that seen with nuclear extracts incubated in vitro with tyrosine phosphatase (22).

The experiments documented that the relative intensity of the TbRC remained virtually the same in controls and in samples immunodepleted of pRb or p107 (Fig. 7A). In contrast, immunodepletion of Sp1 resulted in the disappearance of the TbRC (Fig. 7A). To exclude that loss of TbRC binding might be due to proteolysis, the same Sp1-immunodepleted sample was incubated with Box B. This documented retention of AP1 binding activity and thus inferred integrity of the nuclear extract (Fig. 7B). In conclusion, in vitro treatment of nuclear extracts with protein-tyrosine phosphatase increased the intensity of the TbRC irrespective of p107 and pRb, but not of Sp1. A last immunointerference experiment examined the potential involvement of the Sp1-related inhibitor, Sp3, in TGF-β stimulation of COL1A2 (45). Comparison of the EMSA patterns obtained after preincubation with specific antibodies against Sp3 revealed no difference in relative content of Sp3 between unstimulated and stimulated nuclear extracts (Fig. 8). However, the difference was not as dramatic with the Sp1 antisera and certainly not enough to support the idea of such a
switch in TbRC composition as the major mechanism for COL1A2 stimulation. Altogether, the results of these analyses excluded that availability of active Sp1 and release of Sp3 from the TbRC may play major roles in TGF-β stimulation of COL1A2 gene expression.

DISCUSSION

The pathways leading to TGF-β regulation of genes involved in matrix assembly and remodeling, as well as the transcriptional mechanisms underlying them, are largely unknown. A case in point is the cytokine-induced stimulation of the major structural component of connective tissue, type I collagen. In the past few years, different lines of investigation have yielded conflicting results regarding TGF-β responsiveness to an otherwise unresponsive heterologous promoter. Third, Sp1 competitors or Sp1 antisera eliminate binding of part of the TbRC to the GC-rich sequence of Box 3A. Fourth, in vitro binding of nuclear proteins to the TbRE but neither Box 3A nor Box B alone, increases after TGF-β administration to cell cultures. Fifth, in vivo inhibition of tyrosine phosphatase activity correlates with block of COL1A2 stimulation by TGF-β and with loss of TbRC binding; conversely, inhibition of tyrosine kinase activity has opposite effects.

The alternative proposal hypothesizes that TGF-β stimulates Jun-B expression and this in turn induced COL1A2 transcription by changing the composition of AP1 from Fos/c-Jun to Fos/Jun-B (19). The lines of supportive evidence are the following. First, mutations of the TbRE region suggest that the major responsive sequence resides within a smaller segment of the TbRE comprising nucleotides −265 to −241. Second, AP1 recognizes the −265/−241 sequence in vitro binding assays. Third, the AP1 complex purified from TGF-β-stimulated cells apparently contains Jun-B but not c-Jun. Fourth, overexpression of c-jun in neonatal foreskin cultures inhibits TGF-β stimulation of the −342 promoter. The AP1 model is mostly based on cumulative findings rather than on directly testing how Jun-B overexpression affects COL1A2 expression or what the c-Jun content is in the unstimulated AP1 complex. The former point was addressed in a parallel study of the mouse Col1a2 gene, which indirectly corroborated the AP1 model of TGF-β stimulation (15). The study in fact showed that overexpression of Jun-B in NIH-3T3 fibroblasts increases the activity of the...
co-transfected 350-bp Col1a2 promoter nearly 100-fold. However, the same report also showed that c-jun overexpression stimulated COL1A2 transcription about 4-fold.

We believe that results presented here build a strong and compelling case in favor of the Sp1 over the AP1 model. Addition of mithramycin or curcin to fibroblast cultures revealed that the former (and not the latter) inhibits constitutive and TGF-β-induced transcription of COL1A2. Furthermore, cycloheximide had no effect on TGF-β stimulation of either α2(I) collagen or jun-B mRNA accumulation, thus excluding that induction of the former gene depends on synthesis of the latter product. Together, these results rule out AP1 involvement and bring into question the relevance of the c-Jun to JunB switch. They also point to the strict requirement of GC-rich regulatory elements for proper COL1A2 expression. The conclusion is in line with recent evidence showing that mithramycin addition to human primary fibroblasts reduce constitutive and TGF-β-induced transcription of the coordinately expressed α1(I) collagen gene (46). Moreover, COL1A2 dependence on GC-rich regulatory elements is consistent with recent work that has documented their critical contribution to tissue specificity (9, 12, 20, 28, 46). The causal relationship between the GC-rich binding site of the TbRE and the mithramycin block of COL1A2 stimulation is solidly founded on the independent mapping of the TGF-β-responsive element within this segment of both the human and mouse promoters (13, 18, 19).

The TbRE binds in vitro Sp1 and not AP1. This was demonstrated by a variety of tests, which included immunodepletion and immunodepletion assays using specific Sp1 and AP1 antibodies, as well as treatment with Sp1 and AP1 inhibitors. Collectively, they suggest that AP1 and Sp1 binding to the TbRE are mutually exclusive, and that the latter is normally favored over the former. We have corroborated this claim by documenting the paradoxical responses to PMA of the endogenous gene and of the transfected promoter. Implicitly, the result provides an alternative interpretation of the co-transfection experiments upon which the AP1 model has been built (18). Accordingly, we suggest that the cryptic AP1 sequence overlapping Box B is used under artificial conditions which either eliminate the more favorable binding site (Box B without Box 3A) or force the balance toward the less probable (AP1 overexpression). We cannot, however, exclude the possibility that AP1, alone or in cooperation with the TbRC, may participate in COL1A2 regulation at some later time during TGF-β stimulation, under different physiological or tissue culture conditions, or in different cells.

The above results are also worthy of a few additional comments. The first pertains to the different effect that Sp1 immunodepletion has on TbRC binding compared with the Sp1 immunodepletion and the mithramycin treatment (compare relevant samples of Figs. 4B and 8 with those of Figs. 4C and 7). The difference, loss of Sp1 versus loss of both Sp1 and Cx, may simply reflect experimental idiosyncrasies. Alternatively, it may indicate the distinct requirement of Sp1 for TbRC formation and/or stabilization. In other words, one could reasonably argue that the last two treatments are more effective than immunodepletion in blocking Sp1 availability (immunodepletion) or binding (mithramycin treatment) and, consequently, in eliminating the whole TbRC. The proposal is supported by previous competition experiments, which suggested the existence of stabilizing interactions among the TbRC components, particularly between Sp1 and Cx, the ill defined factor(s) that interacts with Box B (18). Unfortunately, activation of the cryptic AP1 site overlapping Box B has hampered characterization of Cx and thus, clarification of this important point. The second comment regards the discrepancy between the cell transfection data responsible for the formulation of the two opposing models for TGF-β stimulation of COL1A2 transcription (18, 19). Our experiments documented TGF-β responsiveness of a chimeric construct driven by a promoter fragment that includes the AP1 binding site of Box B, but lacks the upstream Sp1 recognition sequence of Box 3A (18). However, the induction was statistically less significant than the one obtained with constructs harboring either Boxes A and B, or Boxes 3A and B (18). Chung et al. (19) reported maximal TGF-β responsiveness with a 5’ deletion construct retaining only the most proximal of the three Sp1 binding sites of the TbRE. We have no explanation for these differences, short of noting that other investigators have also experienced greater loss than Chung et al. (19) in the constitutive activity of the COL1A2 promoter (10-fold versus 2-fold) when mutations were introduced in the Sp1 recognition sequence of Box 3A (20). The third and final comment is in reference to the results of the PMA experiments. Curcin inhibition of COL1A2 down-regulation by PMA indicates that the signal is ultimately transduced through AP1. On the other hand, the responses of the transfected promoter and endogenous gene strongly suggest that the PMA-responsive element of COL1A2 resides outside of the −378 sequence. Together the results infer that AP1 access to the Box B site of the endogenous gene in PMA-treated cells is conceivably hindered by chromatin and/or interactions between the TbRE and other critical regulatory elements, such as the recently described far-upstream enhancer of Col1a2 (47).

The present study has also extended the analysis of the potential factors and mechanisms responsible for TbRC transactivation. Previous work has suggested that tyrosine dephosphorylation of nuclear proteins is an intermediate step in TGF-β stimulation of COL1A2 expression (22). It is, however, unknown if protein dephosphorylation is triggered by increased phosphatase activity or decreased kinase activity. It is also yet to be determined whether the visual change detected by EMSA reflects binding of more TbRC molecules or increased affinity of the prebound complex. Finally, it is yet to be determined what the mechanism in each of these situations might be. We have explored these possibilities as they apply to Sp1, the only known component of the TbRC. Far from being exhaustive, the analysis has nevertheless eliminated some obvious candidates and strengthened our belief that a novel Sp1 partnership is responsible for TbRC transactivation by TGF-β.

In conclusion, we have attempted to resolve the controversy surrounding the identity of transcriptional factors and cis-acting elements and, implicitly, the nature of the molecular mechanisms responsible of the transcriptional response of COL1A2 to TGF-β in cultured fibroblasts. Within the limitations of the in vitro model, we believe that we have presented convincing evidence in favor of our early model. This postulates that modification of an Sp1-containing complex is the last step in the pathway that transduces the TGF-β signal from the cell surface to the COL1A2 gene. We also believe we have convincingly excluded the alternative model, which suggests that the TGF-β signal is elaborated transcriptionally through a change in AP1 composition. In so doing, we have offered an experimental explanation for the discrepancy between the studies supporting each of the two models. We are nevertheless aware of the intrinsic limitations of our method of analysis and of our experimental system, and understand that ultimate proof of the model can only be reached after full biochemical characterization of the TbRC. Work in progress is aimed at achieving this important goal.

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