Cell, Vol. 52, 405–414, February 12, 1988, Copyright © 1988 by Cell Press

A Nuclear Factor 1 Binding Site Mediates the Transcriptional Activation of a Type I Collagen Promoter by Transforming Growth Factor-β

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Summary

Transforming growth factor-β (TGF-β) increases the steady-state RNA levels of several fibroblast extracellular matrix proteins. Using DNA transfection, we show that TGF-β stimulates the activity of the mouse α2(I) collagen promoter 5- to 10-fold in mouse NIH 3T3 and rat osteosarcoma cells. Deletion analysis indicates that a segment of this promoter between −350 and −300, overlapping a nuclear factor 1 (NF1) binding site, is needed for TGF-β stimulation. A 3 bp substitution mutation abolishing NF1 binding to this site inhibits TGF-β activation. Insertion of this NF1 binding site 5′ to the SV40 early promoter makes the promoter TGF-β inducible, but the 3 bp substitution does not. Similarly, when the NF1 binding site at the replication origin of adenovirus 2 and 5 is inserted 5′ to the SV40 promoter, the promoter responds to TGF-β. Therefore an NF1 binding site mediates the transcriptional activation of the mouse α2(I) collagen promoter by TGF-β.

Introduction

Transforming growth factor-β (TGF-β) is a multifunctional peptide regulator of cellular activity (Roberts and Sporn, 1987). It is composed of two disulfide-bonded identical subunits of 112 amino acids that are derived from a much larger precursor polypeptide (Frolik et al., 1983; Assoian et al., 1983; Roberts et al., 1983; Derynck et al., 1985). One striking effect of TGF-β in certain fibroblasts is to stimulate anchorage-independent growth (Roberts et al., 1981). This effect, which served initially as the basis for the purification of the peptide, is seen when TGF-β is either acting alone or is in association with epidermal growth factor (EGF) or TGF-α, depending on the type of cells used in the assay (Shipley et al., 1985; Roberts et al., 1985). In contrast to this role in stimulating cell proliferation, TGF-β also causes growth inhibition of other cell types such as epithelial cells (Moses et al., 1985; Masui et al., 1986; Shipley et al., 1986), endothelial cells (Baird and Durkin, 1986; Fràter-Schröder et al., 1986; Heimark et al., 1986), and B and T lymphocytes (Kehrl et al., 1986a, 1986b). Still another important role of TGF-β is its effect on differentiation of a number of cell types. The hormone prevents differentiation of preadipocytes (Ignotz and Massagué, 1985), myoblasts (Massagué et al., 1986; Florini et al., 1986; Olson et al., 1986), and T and B lymphocytes, whereas it activates the differentiation phenotype of chondroblasts (Rosen et al., 1986; Seyedin et al., 1987).

It has recently become clear that in addition to its role in the growth control of fibroblasts, TGF-β also stimulates the synthesis of several extracellular matrix proteins by these cells, including type I collagen and fibronectin (Ignotz and Massagué, 1986; Roberts et al., 1986; Varga and Jimenez, 1986; Fine and Goldstein, 1987). This effect of TGF-β is observed both with a variety of fibroblasts in culture cells as well as in the intact animal. Indeed, subcutaneous injection of TGF-β in the newborn mouse causes the appearance of a proliferative nodule composed principally of extracellular matrix and fibroblasts (Roberts et al., 1986). Given its role in stimulating the synthesis of extracellular matrix components, it is likely that TGF-β also plays an important role during wound healing (Sporn and Roberts, 1986) as well as in the pathogenesis of various fibrotic diseases.

The diverse cellular roles of TGF-β are presumably all mediated by interactions between the growth factor and specific receptors for the hormone located in the plasma membrane (Massagué and Like, 1985; Massagué, 1985; Cheifetz et al., 1986; Fanger et al., 1986; Cheifetz et al., 1987). However, the components and the nature of the intracellular signaling pathways that are likely to be triggered as a result of the interactions of TGF-β with its receptors are completely unknown.

To investigate the intracellular changes that mediate TGF-β action, we have initiated studies on the mechanism of TGF-β induced increase in synthesis of extracelluiar matrix components by fibroblasts in culture.

Since TGF-β increases the steady-state RNA levels of several extracellular matrix components such as type I, III, and V collagen and fibronectin in fibroblastic cells (Ignotz et al., 1987; Roberts et al., 1988), we have examined whether a chimeric gene in which the α2(I) collagen promoter is fused to a marker gene can be activated in DNA transfection experiments by treatment of fibroblasts with TGF-β. This activation is shown here to be mediated by a specific sequence in the promoter that is a binding site for nuclear factor 1 (NF1).

NF1 was first discovered as a factor required for the in vitro initiation of replication of adenovirus DNA (Nagata et al., 1982, 1983; Rawlins et al., 1984). Biochemical and genetic experiments have indicated that the high-affinity binding of NF1 to a specific site at the origin of replication of the adenovirus genome is essential for DNA replication both in vivo and in vitro (Rawlins et al., 1984; Wang and Pearson, 1985; Hay, 1985; de Vries et al., 1985). NF1 binding sites were later also identified in several viral and cellular promoters (Hennighausen et al., 1985; Nowock et al., 1985; de Vries et al., 1985; Rosenfeld and Kelly, 1986; Schneider et al., 1986). Recently NF1 was shown to stimu-

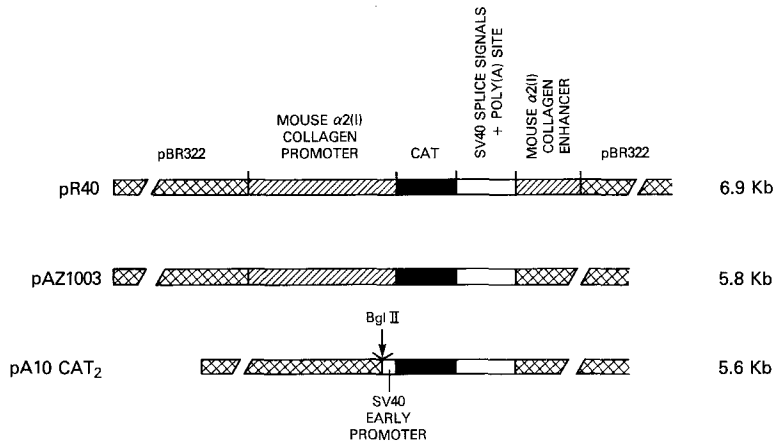


Figure 1. Schematic Representation of Wild-Type Plasmids Used in DNA Transfection Experiments

Plasmid pR40 contains sequences of the mouse α2(I) collagen gene between −2000 and +54, and sequences of this gene present in the first intron between +418 and +1524, which have enhancer activity (Rossi and de Crombrugghe, 1987). The promoter sequences are fused to a segment containing the CAT gene and SV40 processing signals. Plasmid pAZ1003 is identical to pR40 except that it does not contain the mouse α2(I) collagen enhancer sequences (Schmidt et al., 1986). Plasmid pA10CAT$_2$ contains the early promoter of SV40 fused to the CAT gene (Laimins et al., 1984).

late transcription of the α1 globin promoter in vitro (Jones et al., 1987). This cellular factor therefore has a role both in DNA replication and in RNA transcription.

We propose that the control of other genes by TGF-β might also be mediated by NF1 binding sites.

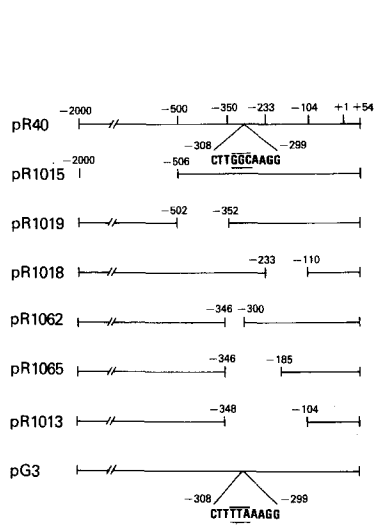
Results

Treatment of several fibroblastic cells such as NRK, mouse NIH 3T3, and rat osteosarcoma cells with TGF-β increases the steady-state RNA levels for several extracellular matrix proteins, including α2(I), α1(III), and α2(V) collagen and fibronectin, between 3 and 6-fold whereas the levels of a control RNA such as the RNA for glyceraldehyde phosphate dehydrogenase are unchanged (Roberts et al., 1988). Ignotz et al. (1987) similarly reported that the levels of fibronectin and α2(I) collagen RNA were elevated after TGF-β treatment of NRK cells and of the mesenchymal rat cell line L_6E_{10}.

TGF-β Activates the Mouse α2(I) Collagen Promoter

To examine whether the increased levels of α2(I) collagen RNA after treatment of fibroblasts with TGF-β could be accounted for by an increased activity of the corresponding promoter, DNA transfection experiments were performed. The chimeric plasmid pR40 used in these experiments contains two important regulatory segments of the mouse α2(I) collagen gene located on each side of the start of transcription (see Figure 1). The first segment, which maps between −2000 and +54 and contains the promoter of this gene, is fused to the gene for chloramphenicol acetyltransferase (CAT). The second collagen fragment, from +418 to +1524, contains a cell-specific transcriptional enhancer located in the first intron of this gene (Rossi and de Crombrugghe, 1987). This fragment is inserted 3′ to the CAT gene and the poly(A) addition site of the vector. Previous transient expression experiments have shown that after DNA transfection of this plasmid, CAT RNA initiates at the same site in the promoter of the chimeric gene as does α2(I) collagen RNA in the endogenous gene (Rossi and de Crombrugghe, 1987).

DNA transfection experiments were performed both in mouse NIH 3T3 fibroblasts and in ROS 17/2 cells, a rat osteosarcoma cell line. All transfections were transient expression experiments in which TGF-β was added after the DNA transfection period. Addition of TGF-β to either NIH



	NIH3T3			ROS17/2		
	RELATIVE CAT ACTIVITY					
	Control	+TGF-β	Ratio	Control	+TGF-β	Ratio
pR40	1.0	7.8	7.8	1.0	5.0	5.0
pR1015	2.9	10.4	3.6			
pR1019	2.2	25.6	11.6			
pR1018	0.4	1.5	3.8			
pR1062	2.4	3.5	1.5	0.4	0.3	0.8
pR1065	0.5	0.6	1.2			
pR1013	0.6	0.7	1.1			
pG3	16.1	21.1	1.3	0.6	0.3	0.5

Figure 2. Effect of Mutations in the α2(I) Collagen Promoter

All plasmids are identical to pR40 except for the indicated deletions or substitutions. Mutant pG3 contains a 3 bp substitution mutation between −305 and −303. The wild-type sequence contains on the lower strand (not shown) a characteristic GCCAA sequence between −303 and −307. CAT activities are expressed relative to the levels of CAT activity obtained with pR40 in the absence of TGF-β. Values represent averages of at least two independent transfection experiments.

Table 1. Specificity of TGF-β

Experiment	Peptide Growth Factor	CAT Activity (cpm [x 10^{-3}] per mg protein)	
		NIH 3T3 Cells	ROS 17/2 Cells
1	None	8.2	2.3
	EGF (5 ng/ml)	11.5	4.0
	Basic FGF (5 ng/ml)	18.7	2.1
	PDGF (5 ng/ml)	13.6	3.4
	TGF-β1 (5 ng/ml)	52.6	14.7
	TGF-β2 (5 ng/ml)	55.5	ND
2	None	3.7	
	EGF (10 ng/ml)	3.2	
	TGF-β1 (5 ng/ml)	14.1	
	EGF (10 ng/ml) + TGF-β (5 ng/ml)	7.8	

Cells were transfected with 10 μg pR40 DNA. ND, not determined; see text for other abbreviations.

3T3 fibroblasts or ROS 17/2 cells transfected with plasmid pR40 causes a marked stimulation in the levels of CAT activity (Figure 2). The concentration of TGF-β needed for half-maximal stimulation of CAT activity in NIH 3T3 fibroblasts is 5 pM (data not shown), which corresponds to the half-maximal concentration needed for other effects of TGF-β (Roberts and Sporn, 1987). It also corresponds to the binding affinity of TGF-β for its receptor(s). A similar concentration of TGF-β for half-maximal stimulation of CAT activity was found after DNA transfection of ROS 17/2 cells (data not shown).

TGF-β also increases the expression of the CAT gene more than 7-fold when an α1(III) collagen promoter is fused to this gene (data not shown). In contrast, with a construct in which the CAT gene is driven by the early promoter of SV40 ($pA10CAT_2$) (see Figure 4A, top line) or by the tyrosine aminotransferase promoter, no significant stimulation of CAT gene expression by TGF-β is observed. Furthermore, although the activity of the α2(I) collagen promoter is about 10 times lower without its enhancer, TGF-β still stimulates this promoter 6 to 7-fold when present in a plasmid from which the enhancer has been removed (data not shown).

Specificity of TGF-β

Table 1 compares the effect of TGF-β and of several other hormones on the level of CAT gene expression when plasmid pR40 (the plasmid containing both the α2[I] collagen promoter and the enhancer of this gene) is used for the transfections. The table shows the results of transient expression experiments in both mouse NIH 3T3 fibroblasts and ROS 17/2 cells. EGF and platelet-derived growth factor (PDGF) show little effect in NIH 3T3 fibroblasts, whereas basic fibroblast growth factor (FGF) shows a 2-fold stimulation of CAT gene expression in these cells. In ROS 17/2 cells, EGF shows some stimulation but clearly less than TGF-β. The effects of TGF-β2 (Cheifetz et al., 1987) on the α2(I) collagen promoter are identical to those of TGF-β1. TGF-β2 is similar in size and biological properties to TGF-β, although the 36 amino-terminal residues of TGF-β2 show only a 69% sequence identity with the same segment in TGF-β. In a parallel experiment performed under the same conditions as the transfection experiments, a cell count was determined 2½ days after the addition of growth factor. The cell number was approximately 70% higher in the presence of either EGF, FGF, TGF-β, or TGF-β2 than in the absence of added growth factors, whereas with PDGF the cell number was twice as high as with the control. We conclude that the effect of TGF-β and of TGF-β2 on the activity of the α2(I) collagen promoter is specific for these hormones and not directly dependent on the effect of TGF-β on cell proliferation. Table 1 also shows that the addition of EGF together with TGF-β decreases the stimulatory effect of TGF-β. It was previously shown that EGF decreases the ability of TGF-β to stimulate collagen synthesis in human dermal fibroblasts and rat NRK cells (Roberts et al., 1986).

Effect of TGF-β on Mutants of the α2(I) Collagen Promoter

To examine which sequences within the α2(I) collagen promoter and upstream regions could be responsible for the effect of TGF-β, several mutations were examined in which portions of the α2(I) collagen promoter and upstream sequences had been deleted (Figure 2). The boundaries of the various deletion mutants, which are all derivatives of pR40 and contain an intact α2(I) collagen enhancer, are shown in Figure 2. It should be noted that for most deletions, the basal level of CAT activity in the absence of TGF-β varies from that observed with the wild-type. With a deletion that removes sequences from −2000 to −500 (pR1015), a stimulatory effect of TGF-β on CAT gene expression is observed, but this stimulation is less pronounced than with the wild-type promoter. In contrast, with a deletion between −500 and −350 (pR1019), the stimulatory effect of TGF-β is more pronounced than with the wild-type promoter. With both deletions the basal level of CAT activity, in the absence of TGF-β, is higher than with the wild-type plasmid. A deletion between −233 and −110 shows a low basal level, but this low basal level can still be induced about 4-fold. However, with all deletions that have position −350 as their approximate 5′ endpoint, the inducibility of TGF-β is strongly reduced. The smallest of these deletions (pR1062) extends between −346 and

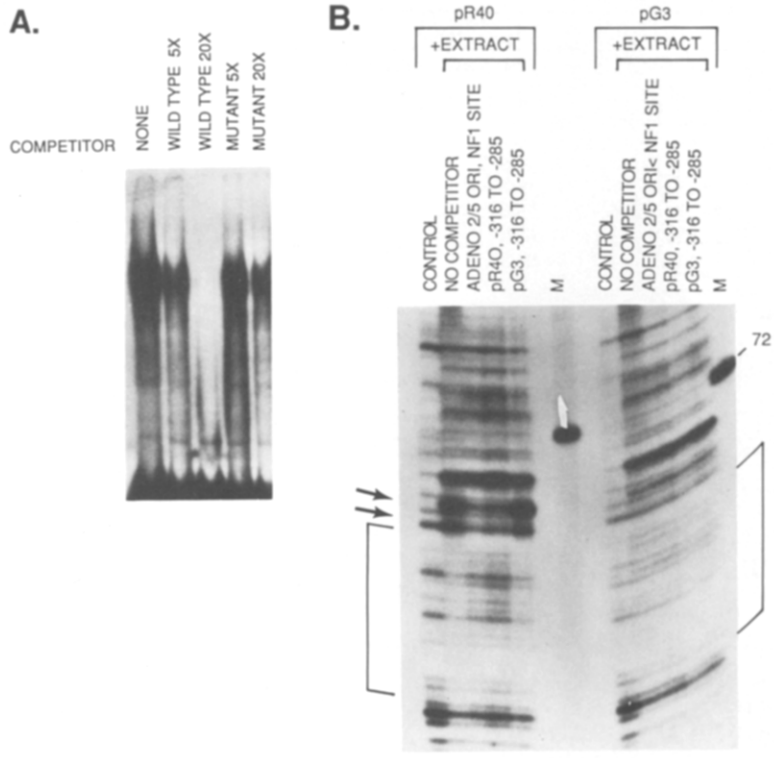


Figure 3. Comparison of NF1 Binding to the Wild-Type and Mutant −300 Segment

(A) Gel retardation assay using a 5′-end-labeled 117 bp BglII–TaqI fragment containing the sequence between −351 and −234, and a crude nuclear extract from NIH 3T3 fibroblasts. Competition experiments were performed with either the same, unlabeled fragment (wild type) or the corresponding mutant fragment. Molar amounts of unlabeled competitor fragment vs. labeled test fragment are indicated.

(B) DNAase I footprint of a 5′-end-labeled 117 bp BglII–TaqI fragment containing sequences between −351 and −234 of either the wild-type α2(I) collagen promoter (pR40) (left) or of the same fragment with a 3 bp substitution from −305 to −303 (pG3) (right). In lanes labeled "+ Extract," DNA was preincubated with a heparin–agarose fraction of rat liver nuclear extract containing NF1 binding activity. Where indicated a 3000-fold molar excess of double-stranded oligonucleotides was added as competitor. The double-stranded oligonucleotides are a 32 bp wild-type sequence (−316 to −285 in the α2[I] collagen promoter) designated pR40, a 16 bp sequence at the origin of replication of adenovirus 2 and 5 (see Figure 4B), and a 32 bp sequence (−316 to −285 in the α2[I] collagen promoter, with a 3 bp substitution mutation as indicated in Figure 4A) designated pG3. Sites that are hypersensitive to DNAase I digestion are indicated with arrows. The area of protection is indicated with brackets.

−300. This deletion is also not inducible by TGF-β after transfection of ROS 17/2 cells. These experiments suggest that an element that lies between −350 and −300 in the α2(I) collagen promoter is responsible for the effect of TGF-β.

In previous experiments using several DNA binding assays, we had observed that a factor present in nuclear extracts of NIH 3T3 fibroblasts and rat liver binds to the segment approximately between −310 and −290 in this promoter (Oikarinen et al., 1987). This factor was identified, on the basis of competition experiments, as NF1. Indeed, binding of a factor present in NIH 3T3 fibroblasts to this sequence was competitively inhibited by a consensus binding site for NF1. The segment between −310 and −290 contains on the template strand of the DNA a sequence 5′-TCGxxxxxGCCAAG-3′, which resembles other NF1 binding sites. To examine the possibility that a binding site for NF1 mediates the effect of TGF-β on the α2(I) collagen gene, we generated by site-specific mutagenesis a 3 bp substitution in the NF1 recognition site of the α2(I) collagen promoter, which changes the wild-type sequence from GCCAAG to TAAAAG.

Two different experiments indicate that this mutation abolishes the binding of NF1. We first performed a gel retardation assay using a 117 bp DNA fragment containing sequences between −351 and −234, and a crude nuclear extract of NIH 3T3 fibroblasts. As we have shown previously, a major retarded band is observed (Figure 3A) that is due to the binding of a factor identified as NF1 (Oikarinen et al., 1987). Excess amounts of wild-type unlabeled (−351 to −234) fragment compete for binding, but the same fragment containing the 3 bp substitution mutation does not. As a source of NF1 we also used a partially purified preparation of NF1 obtained from rat liver. The factor present in this preparation protects the wild-type sequence between −310 and −290 in a DNAase I footprint assay. As was seen previously (Oikarinen et al., 1987), binding of the factor also generates DNAase I hypersensitive sites in this assay. Figure 3B shows that both the protection and the hypersensitivity are strongly decreased by addition of excess amounts of either a double-stranded oligonucleotide containing the NF1 binding site at the origin of replication of adenovirus 2/5 or by a double-stranded oligonucleotide containing the wild-type sequence between −316 and −285 in the mouse α2(I) collagen promoter. No competition is observed with a double-stranded oligonucleotide containing the same sequence with the 3 bp substitution mutation. Furthermore, no protection or hypersensitive sites are detected when the 117 bp fragment (from −351 to −234) from plasmid pG3, which contains the 3 bp substitution, is used in the DNAase I footprint assay. Finally, we have used a completely purified preparation of NF1 (a kind gift from T. Kelly, Johns Hopkins University). In gel retardation assays this factor binds to the wild-type −351 to −234 fragment but not to the mutant fragment (C. Ruteshouser, personal communication).

The plasmid containing this 3 bp mutation was also tested by DNA transfection. As shown in Figure 2, the mu-

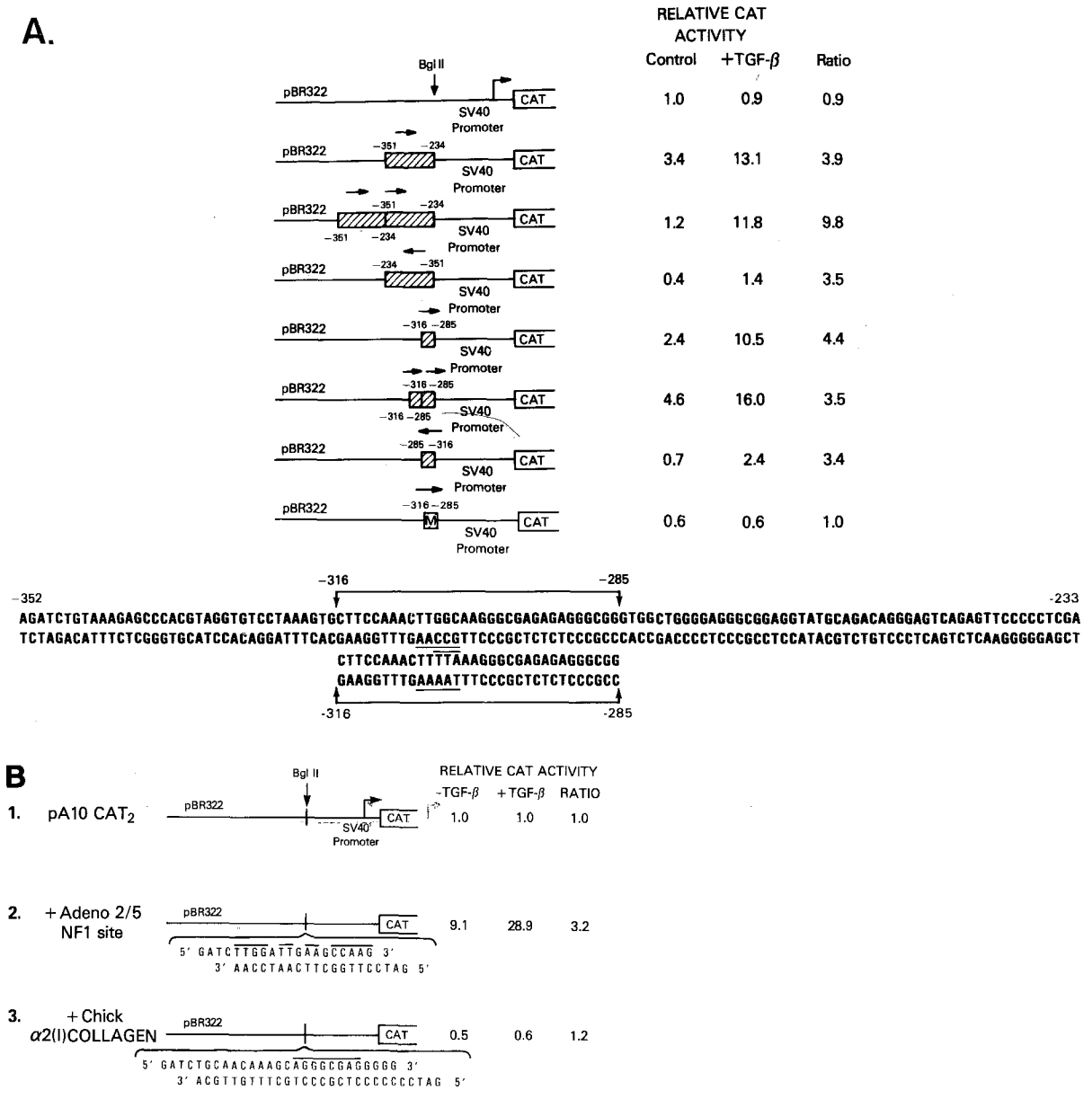


Figure 4. Nuclear Factor Binding Sites Confer TGF-β Inducibility to the SV40 Promoter

(A) The top line represents pA10CAT$_2$. All other plasmids represent constructs in which DNA fragments were inserted into the BglII site of pA10CAT$_2$ 5′ to the SV40 promoter. Horizontal arrows indicate the orientation of fragments in the recombinant plasmids. In the construct shown on the bottom line the −316 to −285 double-stranded oligonucleotide inserted in the BglII site contains the 3 bp substitution mutation present in plasmid pG3 (M). Transfections were performed with 10 μg DNA in NIH 3T3 fibroblasts. Results are expressed relative to the CAT activity obtained with pA10CAT$_2$ in the absence of TGF-β. Values are averages of at least three independent transfection experiments. The sequence between −352 and −233 in the mouse α2(I) collagen promoter is shown below. The fragment between −316 and −285 is bracketed. The sequence inserted into the bottom construct, which contains a 3 bp substitution mutation (indicated by a line above the sequence) in the NF1 site, is also shown.

(B) The NF1 binding site at the origin of replication of adenovirus 2 and 5 confers TGF-β inducibility to the SV40 promoter. Transfections were performed in NIH 3T3 fibroblasts with 10 μg of plasmid DNA. The indicated double-stranded oligonucleotides were inserted into the BglII site of pA10CAT$_2$, 5′ to the SV40 early promoter. The sequence in the chick α2(I) collagen promoter maps between −343 and −323. CAT activities are expressed relative to the CAT activity obtained with pA10CAT$_2$ in the absence of TGF-β.

tation (pG3) virtually eliminates the stimulatory effect of TGF-β on the activity of the α2(I) collagen promoter both in NIH 3T3 fibroblasts and in ROS 17/2 cells. In NIH 3T3 fibroblasts but not in ROS 17/2 cells the mutation produces a surprisingly high basal level of promoter activity in the absence of TGF-β (see Discussion).

The TGF-β Sensitive Segment of the Collagen Promoter Confers TGF-β Inducibility to the SV40 Promoter

We wanted to examine whether the segment of the mouse α2(I) collagen promoter that, by deletion analysis, appears to mediate the stimulatory effect of TGF-β on the α2(I) pro-

moter confers TGF-β dependent inducibility to another promoter that by itself is not stimulated by TGF-β. The DNA segment between −350 and −233 in the mouse α2(I) collagen gene was therefore inserted 5′ to the early promoter of SV40 in the BglII site of pA10CAT$_2$. As is indicated in the first line of Figure 4A, this promoter is not induced by TGF-β. Figure 4A shows that when the −350 to −233 promoter segment is inserted in the same orientation as in the collagen promoter, TGF-β induces the SV40 promoter 4 to 5-fold. The basal level of activity of the promoter also shows an increase. With two copies in the same orientation, the TGF-β induction over the basal level is more pronounced. Furthermore, TGF-β also induces the activity of the SV40 promoter when the same segment is inserted in the opposite orientation.

Similar results were obtained when a smaller segment, containing the sequence between −315 and −284 in the α2(I) collagen promoter, was inserted in the BglII site of pA10CAT$_2$, 5′ to the SV40 promoter. This segment corresponds to the site protected by NF1 in DNAase I footprint experiments plus a few base pairs on each side of the protected area (Oikarinen et al., 1987). As shown in Figure 4A, TGF-β increases the activity of the SV40 promoter when this fragment is cloned in either orientation or when two copies of the fragment are inserted, although the basal level is lower in the opposite than in the direct orientation. However, when the same −315 to −284 fragment containing the 3 bp substitution mutation of pG3 was inserted in the BglII site of pA10CAT$_2$, TGF-β failed to produce any increase in CAT activity (last line of Figure 4A). These experiments indicate that the segment in the α2(I) collagen promoter that we previously had identified as a binding site for NF1 confers TGF-β inducibility to another promoter.

NF1 was initially identified as a cellular factor stimulating in vitro replication of adenovirus DNA (Nagata et al., 1983). A specific binding site for NF1 was demonstrated at the origin of DNA replication in the adenovirus genome. This site is essential for adenovirus DNA replication both in vitro and in intact cells (de Vries et al., 1985; Hay, 1985; Wang and Pearson, 1985). To test whether this sequence would also confer TGF-β inducibility to the SV40 early promoter, we inserted a 16-mer synthetic double-stranded oligonucleotide, corresponding to the NF1 binding site in the adenovirus origin of replication, 5′ to the early SV40 promoter. As shown in Figure 4B, DNA transfection with this construct leads to an increase in the basal level of activity of the SV40 promoter. Treatment of cells with TGF-β clearly induces the activity of this promoter at least 3-fold more.

As an additional control for the experiments shown in Figures 4A and 4B, another double-stranded oligonucleotide was cloned into the BglII site of pA10CAT$_2$. This oligonucleotide contains the sequence of a segment of the chick α2(I) collagen promoter between −343 and −323. This segment is partially homologous with the mouse sequences between −315 and −285, but the chick sequence does not show any homologies with a nuclear factor binding site. Figure 4B shows that TGF-β is unable to stimulate CAT gene expression with this construct is transfected into NIH 3T3 fibroblasts. With this construct the basal level of CAT activity is also not increased above the control level.

Discussion

TGF-β Causes Transcriptional Activation of the α2(I) Collagen Promoter

The results of our DNA transfection experiments demonstrate that treatment of fibroblasts and osteosarcoma cells with TGF-β causes an activation of the promoter of the α2(I) collagen gene. This transcriptional activation could account in large part for the increased collagen synthesis and the increased steady-state levels of type I collagen RNAs observed after treatment of fibroblasts with TGF-β.

The two types of cells that we have used for the DNA transfection experiments, NIH 3T3 fibroblasts and ROS 17/2 cells, show different proliferative responses to TGF-β. Whereas TGF-β stimulates the growth of NIH 3T3 fibroblasts under the conditions of the transient expression experiments, it has no or very little effect on the growth of ROS 17/2 cells under these conditions. Yet the effect of TGF-β on the activity of the wild-type α2(I) collagen promoter and on mutants of this promoter is similar with the two kinds of cells. It is therefore unlikely that the increased CAT gene expression in TGF-β treated NIH 3T3 fibroblasts is a nonspecific consequence of the growth stimulation of these cells by TGF-β. Other peptides that stimulate the growth of these cells, such as FGF, EGF, and PDGF, have little effect on the activity of the α2(I) collagen promoter. Finally, the specificity of activation of the α2(I) collagen promoter is shown by the fact that the SV40 promoter is not stimulated by TGF-β in either NIH 3T3 fibroblasts or ROS 17/2 cells and by the fact that specific mutations in the α2(I) collagen promoter abolish the effect of TGF-β.

An NF1 Binding Site Mediates the TGF-β Induced Stimulation

A deletion of about 50 bp between −346 and −300 strongly decreases the TGF-β inducibility of this promoter. Our previous experiments have shown that nuclear extracts of NIH 3T3 fibroblasts contain a factor that specifically binds to the sequence between −310 and −290. This sequence, which is presented in Figure 4A, contains an NF1 binding site on the template strand. Competition experiments showed that the factor present in nuclear extracts of NIH 3T3 fibroblasts that binds to this sequence is, in fact, NF1 (Oikarinen et al., 1987). It should be noted that this factor is distinct from another factor, which binds to a CCAAT sequence between −84 and −80 in the same promoter (Oikarinen et al., 1987).

Several lines of evidence indicate that the sequence in the α2(I) collagen promoter that mediates the effect of TGF-β in the activation of this promoter is a binding site for NF1. First, a 3 bp substitution mutation in the binding site for NF1 abolishes the TGF-β inducibility of the α2(I) collagen promoter. This same mutation also prevents the binding of NF1 in DNA binding assays. Second, the sequence between −310 and −270 was used in DNA affinity chromatography to purify factors present in rat liver nu-

clear extracts that bind to this sequence. The SDS–polyacrylamide gel electrophoresis analysis of the proteins isolated by this method shows a pattern of four to five polypeptides with masses between 54,000 and 65,000 daltons (J. Oikarinen, personal communication). This pattern is very similar to the pattern reported by others for purified NF1 (Rosenfeld and Kelly, 1986; Jones et al., 1987). Third, a 30 bp segment of the α2(I) collagen promoter between −315 and −285, which centers around the NF1 binding site, confers TGF-β inducibility to the SV40 promoter, a promoter that is not responsive to TGF-β. The same segment containing a 3 bp substitution, which inhibits NF1 binding in DNA binding assays, is unable to confer TGF-β inducibility. Finally, a 16-mer oligonucleotide containing the well-characterized NF1 binding site located at the origin of replication of the DNA of adenovirus 2 and 5, a site essential for adenovirus DNA replication, confers TGF-β inducibility to the same SV40 promoter when this sequence is placed upstream of the viral promoter. A control oligonucleotide that does not contain an NF1 binding site is unable to confer TGF-β inducibility to the SV40 promoter when it is inserted in the same place upstream of the promoter.

TGF-β also stimulates the activity of a construct containing the α1(III) collagen promoter. This plasmid contains a segment of the mouse α1(III) collagen gene between −80 and +16 that is fused to the CAT gene. We have noted that this α1(III) collagen sequence contains a typical NF1 recognition sequence around −50 (Liau et al., 1985). We speculate that the activation of this and other promoters such as those for the fibronectin gene, the α1(I) collagen gene, and possibly many other genes is also mediated by NF1 binding sites.

It should be noted that TGF-β may have additional effects on the expression of the type I collagen genes. A recent report presented data that were interpreted as suggesting that TGF-β also increases the stability of the RNAs for type I collagen (Raghow et al., 1987).

Mutations in the α2(I) Collagen Promoter

The effect of TGF-β on the different deletion mutants of the α2(I) collagen promoter varies from deletion to deletion. Removal of the segment between −2000 and −500 lowers the extent of the induction by TGF-β, whereas a deletion of the sequence between −500 and −350 causes a more pronounced stimulation in CAT gene expression by TGF-β than is observed with the wild-type plasmid. Still, with another deletion, removing the sequence between −233 and −104, a lower TGF-β inducibility is seen. This mutant shows a low basal level of expression in the absence of TGF-β. These deletions might remove interaction sites for DNA binding proteins that could influence the level of activity of the promoter.

The 3 bp substitution mutation in the NF1 recognition site, around −300 in the α2(I) collagen promoter, leads to a surprisingly high level of CAT gene expression in NIH 3T3 fibroblasts in the absence of TGF-β. One possible interpretation for this high basal level is that the mutation has altered the DNA binding site for a repressor molecule, and hence that NF1 may act as a negative regulator of the collagen promoter. It is also possible that two different regulatory factors, one a repressor molecule and the other an activator (such as NF1), bind to the same sequence or overlapping sequences (for instance, on two different faces of the helix). The 3 bp substitution mutation could alter the binding of both molecules. Alternatively, one might consider that the 3 bp substitution has created a new site for a positive factor and that interactions between this new site and a putative positive factor would account for the high basal level. This high basal level found in NIH 3T3 cells is, however, not observed after transfection of the 3 bp substitution mutation into ROS 17/2 cells. This would suggest that NF1 does not act as a repressor in the control of the α2(I) collagen promoter in these cells. The fact that the SV40 early promoter becomes inducible by TGF-β after the insertion of an NF1 recognition site 5′ to the viral promoter suggests that NF1 acts, in fact, as a positive factor.

The stimulation of the α2(I) collagen and other promoters by TGF-β could be mediated either directly by NF1 itself or by another transcriptional factor that interacts with NF1. Alternatively, TGF-β treatment of cells might result in removal of a factor that inhibits the activity of NF1. We have not yet determined whether TGF-β produces an increase in the synthesis of NF1 or a modification of NF1, or whether TGF-β increases the synthesis of or alters another factor that itself interacts with NF1. However, since NF1 acts both as a transcriptional factor and a DNA replication factor, it is tempting to speculate that NF1 itself mediates both the changes in gene expression and the other effects of TGF-β such as those related to cell proliferation. A multifunctional polypeptide such as TGF-β might have a correspondingly multifunctional intracellular mediator such as NF1.

Experimental Procedures

DNA Constructs

The details of the construction of several plasmids carrying deletions in the promoter region of the mouse α2(I) collagen gene have been reported elsewhere (Schmidt et al., 1986). In this series of plasmids a 1.1 kb BamHI fragment, containing sequences of the first intron of the mouse α2(I) collagen gene (from position +418 to +1524 relative to the transcriptional start site), was inserted in the direct orientation into the BamHI site 3′ to the poly(A) addition site in these plasmids. This fragment contains a cell-specific transcriptional enhancer (Rossi and de Crombrugghe, 1987). In the series of $pA10CAT_2$ chimeric constructs, the inserts were cloned in the BglII site of $pA10CAT_2$ (Laimins et al., 1984), immediately upstream of the enhancerless early SV40 promoter. These constructs were used to localize the sequences within the collagen promoter that are needed for transcriptional induction by TGF-β. To clone the fragment of the mouse α2(I) collagen gene containing sequences between −350 and −233, pAZ1003 (Schmidt et al., 1986) was first digested with TaqI, followed by treatment with T4 polymerase to make the ends blunt and addition of BglII linkers. The DNA was subsequently digested with BglII, and the 120 bp BglII fragment was isolated and cloned into the BglII site of $pA10CAT_2$. The four double-stranded synthetic oligonucleotides cloned in $pA10CAT_2$ contained a BglII cohesive end on the 5′ side and a BamHI cohesive end on the 3′ side of the coding strand. After hybridization, the complementary oligonucleotides were phosphorylated at their 5′ ends with T4 polynucleotide kinase and ligated to the $pA10CAT_2$ vector, which had been linearized with BglII and treated with alkaline phosphatase. The copy number and

the orientation of the inserts were determined by restriction analysis. All plasmids were grown in Escherichia coli N38 and banded twice in cesium chloride. Plasmid preparations contained less than 5% RNA and consisted essentially of supercoiled DNA, as assayed by agarose gel electrophoresis.

DNA Transfections

Mouse NIH 3T3 fibroblasts were routinely maintained in DMEM containing 10% calf serum. ROS 17/2 cells (a kind gift of Dr. Luisa Brandi, NIH) were maintained in a 1:1 mixture of DMEM and HAM F12 containing 5% fetal bovine serum. Both cell lines were kept in 8% CO_2 and plated approximately 15 hr before transfection at a density of 0.7 to 0.9 $\times$ 10^6 cells in 10 cm diameter plastic dishes. Transfections were performed essentially as previously described (Schmidt et al., 1985) using 10 μg of plasmid DNA in 1 ml of a $CaPO_4$ coprecipitate. Four hours after DNA addition, the cells were treated with glycerol and the medium was replaced by DMEM without serum. Four hours later hormones were added at the concentrations indicated in the figure legends. A single suspension of DNA coprecipitate was used each time for both the TGF-β and control dishes. TGF-β was resuspended in a solution containing 4 mM HCl and 10 mg/ml BSA. An equal volume of the carrier solution was added to the control plates. Cells were harvested 40 to 42 hr after transfection and assayed for CAT activity as previously described (Gorman et al., 1983). CAT activities were expressed as cpm of acetylated chloramphenicol per mg protein per hr. Approximately 1 $\times$ 10^6 cpm was input per assay.

In the experiments reported here, serum was removed from the culture medium before addition of TGF-β. This was done to examine the effects of the growth factor in the absence of other hormones normally present in serum. However, the effect of TGF-β is still detectable in the presence of either 0.1% or 1% calf serum. In NIH 3T3 fibroblasts but not in ROS 17/2 cells, the levels of CAT activity in the absence of TGF-β increase when serum is present, although the absolute level of CAT activity after TGF-β treatment remains approximately the same. We suspect that ROS 17/2 cells and NIH 3T3 fibroblasts respond differently to the balance of factors present in serum that act positively and negatively to influence the α2(I) collagen promoter.

RNA Analysis

RNA was isolated from control and treated cells by the guanidine thiocyanate–cesium chloride method (Chirgwin et al., 1979). The concentration of the RNAs was determined from the absorption at 260 nm. The integrity and concentrations of the RNAs were verified by formaldehyde–agarose denaturing gel electrophoresis. For the Northern hybridization experiments 2–10 μg of total RNA was fractionated on 1% agarose–formaldehyde gels and transferred to Nytran membranes according to the directions of the manufacturer. Hybridizations were performed for 18 hr at 42°C in 50% formamide. Filters were washed at 65°C in 0.1× SSC, 0.1% SDS. For the primer extension assays 50 μg of total RNA was hybridized with an excess of a 5′-end-labeled, single-stranded antisense oligonucleotide complementary to the sequences from +73 to +96 in the mouse α2(I) collagen RNA. The hybridization was performed at 50°C for 8 hr. The conditions for reverse transcription and electrophoretic analysis of the cDNA products (6.5% acrylamide–7 M urea gels) were as previously described (Schmidt et al., 1986).

Generation of Plasmid pG3

Site-specific mutagenesis was performed according to the method of Kunkel (1985). Briefly a 240 bp BglII–SmaI fragment of pR40 was inserted into the replicative-form DNA of M13mp19 phage. Uridine-containing phage DNA was produced by growing the recombinant phage in *dut*$^-$ *ung*$^-$ E. coli. A 30 bp synthetic oligonucleotide containing a 3 bp substitution mutation in the NF1 binding site was hybridized with single-stranded phage DNA and extended with DNA polymerase I. The product of the reaction was used to transform competent *dut*$^+$ *ung*$^+$ E. coli. Recombinant plaques were screened by dideoxy sequencing. After isolation of replicative-form DNA corresponding to a positive plaque, the mutated BglII–SmaI fragment was reinserted into the original pR40 vector in place of the wild-type BglII–SmaI fragment, generating plasmid pG3.

Hybridization Probes

The mouse α2(I) collagen probe is an 850 bp XhoI–EcoRI fragment from pAZ1003 (Schmidt et al., 1986). The human α1(III) collagen probe is a 1 kb PvuIII–PvuI insert of plasmid RJ5 (Loidl et al., 1984). The human α2(V) collagen probe is a 1.4 kb PstI insert of plasmid NH20 (Myers et al., 1985). The rat fibronectin probe is a 0.5 kb EcoRI fragment from λrlf1 (Schwarzbauer et al., 1983). Probes were labeled with ^{32}P by nick translation.

DNAase I Footprints

DNA fragments were obtained from plasmids pR40 and pG3. The plasmids were digested with BglII, treated with alkaline phosphatase (from calf intestine), labeled with [γ-^{32}P]ATP, and digested with TaqI. The 117 bp fragments (corresponding to sequences from −350 to −233 in the promoters) were isolated on nondenaturing acrylamide gels, extracted in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS, and concentrated by ethanol precipitation. DNA binding reactions were performed in a final volume of 50 μl in 60 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 mM Tris–HCl (pH 7.5), 0.5 mM DTT, 5% glycerol (WN), for 20 min at room temperature with 0.5 ng (~5000 cpm) of labeled fragment, in the presence of 50 ng (HaeIII-digested) ΦX174 DNA and 20 μg tRNA. Samples were incubated either with or without 15 μg protein of a nuclear extract prepared from rat liver nuclei. The extract had been partially purified on heparin–agarose, and concentrated and desalted by PEG precipitation (a kind gift of Paul Golumbek, The University of Texas System Cancer Center). DNAase I digestion was performed at room temperature for 90 min by addition of 5 μl of a solution containing 0.1 M $MgCl_2$ and 0.1 M $CaCl_2$, and 5 μl of a 1:750 dilution from a 1 mg/ml stock solution of DNAase I (Worthington), freshly prepared in 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mg/ml BSA. The same amount of DNAase I was used in control samples and in samples preincubated with the extract either in the presence or absence of competitor double-stranded oligonucleotides. Reactions were stopped with 10 μl of 5% SDS, 0.125 M EDTA, extracted twice with phenol–chloroform, ethanol precipitated, washed with 80% ethanol, dried, resuspended in 95% formamide buffer, heat denatured, and loaded on a 6% acrylamide–7 M urea gel. Gels were fixed, dried, and exposed at −70°C for 2 days with intensifying screens.

Growth Factors

All experiments were performed with TGF-β1 unless noted otherwise. Human TGF-β1 was purified from platelets (Assoian et al., 1983). Porcine TGF-β2 was obtained from R&D Systems (Minneapolis, MN). Murine EGF was purified as described by Roberts et al. (1981). Porcine PDGF was obtained from Bethesda Research Laboratories, and bovine FGF was a gift from Andrew Baird (Salk Institute).

Acknowledgments

We thank Paul Golumbek and Shinichi Watanabe for preparing the oligonucleotides, Maria Mudryj for preliminary experiments and for the plasmid containing the α1(III) collagen promoter, Jeanne Myers (University of Pennsylvania) for human α1(III) and α2(V) collagen probes, R. O. Hynes (MIT) for a human fibronectin probe, and Joyce Sharrar at NIH and Janie Finch at M. D. Anderson Hospital for editorial assistance. P. R. is the recipient of an Arthritis Foundation fellowship, and on leave of absence from the Department of Public Health and Cell Biology, 2nd University of Rome, Tor Vergata, Via Orazio Raimondo, Localita La Romanina, 00173, Rome, Italy.

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Received July 10, 1987; revised November 23, 1987.

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