Tenascin-C Expression by Fibroblasts Is Elevated in Stressed Collagen Gels

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Abstract. Chick embryo fibroblasts cultured on a collagen matrix exert tractional forces leading to the contraction of unrestrained, floating collagen gels and to the development of tension in attached, restrained gels. On a restrained, attached collagen gel the fibroblasts synthesize large quantities of tenascin-C, whereas in a floating, contracting gel tenascin-C synthesis is decreased. This regulation of tenascin-C synthesis can be observed by the secretion of metabolically labeled tenascin-C into the conditioned medium, as well as by the deposition of tenascin-C into the collagen matrix as judged by immunofluorescence. Regulation appears to occur at the transcriptional level, because when cells on attached or floating collagen gels are transfected with promoter constructs of the tenascin-C gene, luciferase expression driven by the tenascin-C promoter parallels the effects measured for endogenous tenascin-C synthesis, whereas luciferase expression under the control of the SV40 promoter does not depend on the state of the collagen gel. The promoter region responsible for tenascin-C induction on attached collagen gels is distinct from the region important for the induction of tenascin-C by serum, and may define a novel kind of response element. By joining this tenascin-C sequence to the SV40 promoter of a reporter plasmid, its activity can be transferred to the heterologous promoter. We propose that the tenascin-C promoter is directly or indirectly activated in fibroblasts generating and experiencing mechanical stress within a restrained collagen matrix. This may be an important aspect of the regulation of tenascin-C expression during embryogenesis as well as during wound healing and other regenerative and morphogenetic processes.

One of the most striking features of the extracellular matrix protein tenascin-C is its dynamically changing expression pattern during embryogenesis (Erickson and Bourdon, 1989; Prieto et al., 1990). However, relatively little is known about the regulation of tenascin-C expression. So far various growth factors, such as transforming growth factor-β (TGF-β)[1] (Pearson et al., 1988; Chiquet-Ehrismann et al., 1989), bFGF (Tucker et al., 1993), activin (Umbhauer et al., 1992), platelet-derived growth factor and angiotensin II (Mackie et al., 1992), several different serum fractions (Vručinić-Filipi and Chiquet-Ehrismann, 1993), as well as various combinations of cytokines (Rettig et al., 1994) have been shown to be able to induce tenascin-C synthesis. Furthermore, glucocorticoids were very recently reported to down-regulate tenascin-C expression (Ekblom et al., 1993). Each of these studies used different experimental systems and very different cell types, and it has been shown that different types of cells respond differently to the same cytokines (Rettig et al., 1994). These reports show that expression of tenascin-C can easily be experimentally manipulated.

To delineate the mechanisms of regulation of transcription of the tenascin-C gene, the tenascin-C promoter region has been cloned and was used for transient transfection studies (Jones et al., 1990). It was shown that serum could induce the expression of reporter constructs under the control of the tenascin-C promoter. Interestingly, the tenascin-C promoter constructs could also be activated by cotransfection of cells with the homeobox-containing gene *Evx-1*, although the activation appeared to be indirect, triggering a growth-factor signal transduction pathway rather than via direct activation of the tenascin-C promoter by this transcription factor (Jones et al., 1992).

We have found in earlier studies that in high density limb bud cultures, elevated tenascin-C expression accompanied the formation of cartilage nodules (Mackie et al., 1987). The deposition of tenascin-C in such cultures showed an interest-

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1. *Abbreviations used in this paper*: API, activator protein 1; NF, nuclear factor 1; TGF-β, transforming growth factor-β.

ing pattern: it was high around the developing cartilage nodules themselves and also outlined the tension lines which formed between the condensing nodules. This led us to speculate that mechanical stress could induce the local production and deposition of tenascin-C.

In this paper we investigated the possibility that tenascin-C transcription could be influenced by mechanical forces which cells generate and experience under different culture conditions. It is known that fibroblasts cultured within collagen gels contract such gels as a result of tractional forces exerted by the cells which attach to the collagen fibers (Bell et al., 1979; Stopak and Harris, 1982). If the gel is anchored to the culture dish, it resists pulling by the fibroblasts and the cells experience mechanical strain equivalent to the tractional force which they generate (Stopak and Harris, 1982). Strain gauge measurements showed that the force generated by fibroblasts in collagen gels is comparable to that observed in contracting skin wounds (Delvoye et al., 1991; Kolodney and Wysolmerski, 1992). The force generated by chick embryo fibroblasts was measured to be 4.5×10^4 dynes/cm^2 and required an active cytoskeleton (Kolodney and Wysolmerski, 1992). The traction is transduced to the collagen matrix through β1 integrins (Gullberg et al., 1990; Chan et al., 1992). On a floating collagen gel, the cells slowly contract the gel isotonically, and strain is low. We therefore tested whether tenascin-C expression by chick embryo fibroblasts is affected by the mechanical state of collagen gels on which they are cultured.

Materials and Methods

Metabolic Labeling of Fibroblasts in Collagen Gels

11-d-old embryonic chick skin fibroblasts were cultured in collagen gels as described by Stopak and Harris (1982). Briefly, 5 parts collagen solution (Sigma type III from calf skin, 3.2 mg/ml in 30 mM acetic acid) were mixed at 4°C with 2 parts 5× DMEM (GIBCO BRL, Gaithersburg, MD), 1 part 150 mM NaOH, 0.5 parts FCS (GIBCO BRL), and 1.5 parts Dulbecco's PBS. This solution (2 ml) was added to a pellet containing 10^6 chick embryo fibroblasts, mixed, and poured into 35-mm culture dishes. These dishes had previously been covered with silicon rubber (Silgard; Dow Corning, Seneffe, Belgium), and either did or did not contain four plastic poles, arranged in a square and glued onto the dish with epoxy glue (Devcon Corp., Danvers, MA). Dishes were incubated at 37°C and a collagen gel with embedded cells formed within 20 min. Within 2 d, the fibroblasts contracted the gel either to a small clump (unrestrained), or to a sheet suspended between the plastic poles (restrained; see Fig. 1).

After 36 h in culture, cells plated on plastic and on unrestrained and restrained cultures were washed twice with methionine-free medium, and 1.5 ml ^{35}S-cysteine/methionine (2:1) in methionine-free DMEM/5% FCS was added. To some cultures on restrained gels 2 μg/ml of cytochalasin (Sigma Chem. Co., St. Louis, MO) was added 12 h before labeling. After 20 h of labeling, the conditioned medium was collected. The collagen gels containing the cells were washed twice with 150 mM NaCl, 20 mM Tris–HCl, pH 7.4, and then extracted with 1.5 ml 2% Na-deoxycholate, 2 mM EDTA, 5 mM *N*-ethyl maleimide, 2 mM phenylmethylsulfonyl fluoride, 50 mM Tris–HCl, pH 8.0.

Conditioned media and deoxycholate extracts were analyzed by two-dimensional SDS-PAGE (nonreduced vs. reduced) as described by Koch et al. (1992). Samples (120 μl) were run in the first dimension on 3–10% acrylamide gradient gels without prior reduction. Lanes were cut, placed horizontally on top of a second 3–15% acrylamide gradient gel, overlaid with reducing sample buffer, and run in the second dimension. Gels were dried and fluorographed. Disulfide-linked oligomeric proteins are resolved into spots beneath the diagonal of the gel. Tenascin-C hexamers and trimers were identified by comigration with purified unlabeled protein as described in Chiquet et al. (1991).

Cell Cultures for Immunofluorescence

Collagen gels were produced from acid soluble rat tail collagen (Sigma Chem. Co.). The collagen was dissolved in 0.1% acetic acid at 3 mg/ml. The solution was kept at 4°C. It was neutralized using 0.34N NaOH and 1/10 vol of 10-fold concentrated culture medium was added. Now 10^7 cells/ml of collagen solution were included and the collagen solution was pipetted into culture wells (96-well clusters; Falcon). The plates were incubated for 30 min in the 37°C CO_2 incubator during which time the collagen gel polymerized. After polymerization, medium containing 0.1% FCS was added and some of the gels were detached from the culture dish. Two days later the attached as well as the detached, contracted collagen gel cultures were fixed, sectioned and stained as described by Mochitate et al. (1993). The antibodies used were chicken-specific monoclonal antibodies against tenascin-C and fibronectin as described (Pearson et al., 1988).

Isolation and Production of the Tenascin-C Promoter Constructs

From a genomic library prepared from adult chicken liver DNA (Clontech, Palo Alto, CA) we isolated a phage containing 4 kb of 5′ sequences of the transcription start site, the first exon as well as about 7.5 kb of the first intron of the tenascin-C gene. The 4 kb of promoter sequence has been published (Jones et al., 1990) and is available in the EMBL database under the accession number M35369. The 4 kb of promoter region as well as 44 bp of the first intron were subcloned and inserted in the reporter plasmid pPALU (Artelt et al., 1991) in front of the luciferase gene, joining the first 44 bp of the first tenascin-C exon to the first exon of the luciferase resulting in the plasmid p40Tn. Using convenient restriction sites, the following shorter constructs were prepared: p12Tn, p06Tn, and p02Tn, containing 1215, 565, and 202 bp of the tenascin-C promoter, respectively. The promoter sequences contained in these constructs are seen in Fig. 6. This sequence corresponds to the database entry M35369 from position 2809–4065, with the exception of eight nucleotide exchanges or insertions, (A to C at −704; T to C at −689; insertion of C at −565; T to C at −481; A to G at −477; C to T at −431; insertion of a T at −259; G to A at −30; according to the numbering in Fig. 6). The additional cytidine at position −565 led to the presence of the restriction site for Eco47III, which was used for the construction of p06Tn as well as for the construction of the Ball deletion clones. A plasmid containing the p12Tn promoter sequence was cut by Eco47III and digested by Ball. After different time intervals the nuclease reaction was stopped, the DNA relegated and a representative set of deletion clones were selected based on their size, sequenced and used for the transfection experiments. We analyzed the six clones p12Tn/b1–b6. The exact sequences of each of these clones can be seen in Fig. 6. To construct the hybrid promoter constructs pTn1-SV40 and pTn2-SV40 we inserted the segments from nucleotide number −570 to −202 or −570 to −469, respectively, in 5′ position of the SV40 promoter in the plasmid pPALU. For cotransfection to normalize for the transfection efficiency we used a RSV-β-gal reporter plasmid.

Transfection Procedure

Chick embryo fibroblasts were isolated from the skin of 11-d-old embryos. The F0 generation was propagated and stored in aliquots under liquid nitrogen. Each experiment was done using a fresh aliquot of these primary cultures. Chick embryo fibroblasts were thawed and plated in large culture plates in Dulbecco's medium (Amimed; BioConcept, Allschwil, Switzerland) containing 10% FCS for 4 d. For the transfection experiment, they were subcultured at 0.5×10^5 cells/well in medium containing 10% FCS into 24-well plates (Falcon Plastics, Cockeysville, MD) that did or did not contain collagen gels (250 μl of collagen per well). After one day the medium was removed and replaced by medium containing 0.03% FCS. Three times every half hour the medium was replaced to ensure that the higher amount of FCS was washed out of the collagen gels. The cells were transfected using 1.6 μg of DNA and 4 μl of DOTAP (Boehringer Mannheim Corp., Indianapolis, IN) in 0.5 ml of medium containing 0.03% FCS per well. When using cotransfection with the RSV-β-gal reporter plasmid we used 0.8 μg of each plasmid per well. After 16 h, the transfection medium was removed and fresh medium containing the FCS concentrations indicated in each experiment was added. The collagen gels were either left untouched (attached collagen gel) or they were lifted off the culture dish using a spatula (floating collagen gel). 60 h later the medium was aspirated and the cells were collected in lysis buffer (0.1 M H_2KPO_4/HK_2PO_4, pH 7.8; 0.5% Triton X-100; 1 mm DTT). After repeated freezing and thawing, the

lysates were centrifuged and the luciferase activity in the supernatant was assayed in a LKB Bio-Illuminator using the following assay buffer: 25 mM glycine, pH 7.8; 5 mM ATP; 15 mM $MgSO_4$; 0.3 mM luciferin (Sigma Chem. Co.).

Results

To detect proteins secreted by chick embryo fibroblasts that may be induced under conditions of mechanical stress, we analyzed the metabolically labeled proteins of cells cultured in restrained versus nonrestrained collagen gels. Photographs of these cultures are shown in Fig. 1. While at the start of the culture the collagen matrix covered the entire dish, after two days the cells in the nonrestrained (floating) gels contracted the matrix to a small disc (Fig. 1 *d*). In cultures restrained with plastic poles, the partially contracted gel had the shape of a stretched rubber sheet (Fig. 1 *a*). Since we were mostly interested in analyzing extracellular matrix proteins, many of which occur as disulfide-linked oligomers, we separated cell extracts and conditioned medium on two-dimensional gels, where the first dimension was run under nonreducing conditions and the second dimension under reducing conditions. This allows the disulfide-linked subunits of extracellular matrix proteins to be seen as isolated spots below the diagonal containing the majority of the proteins. The result is shown in Fig. 1, *b*, *c*, *e*, and *f*. Tenas-

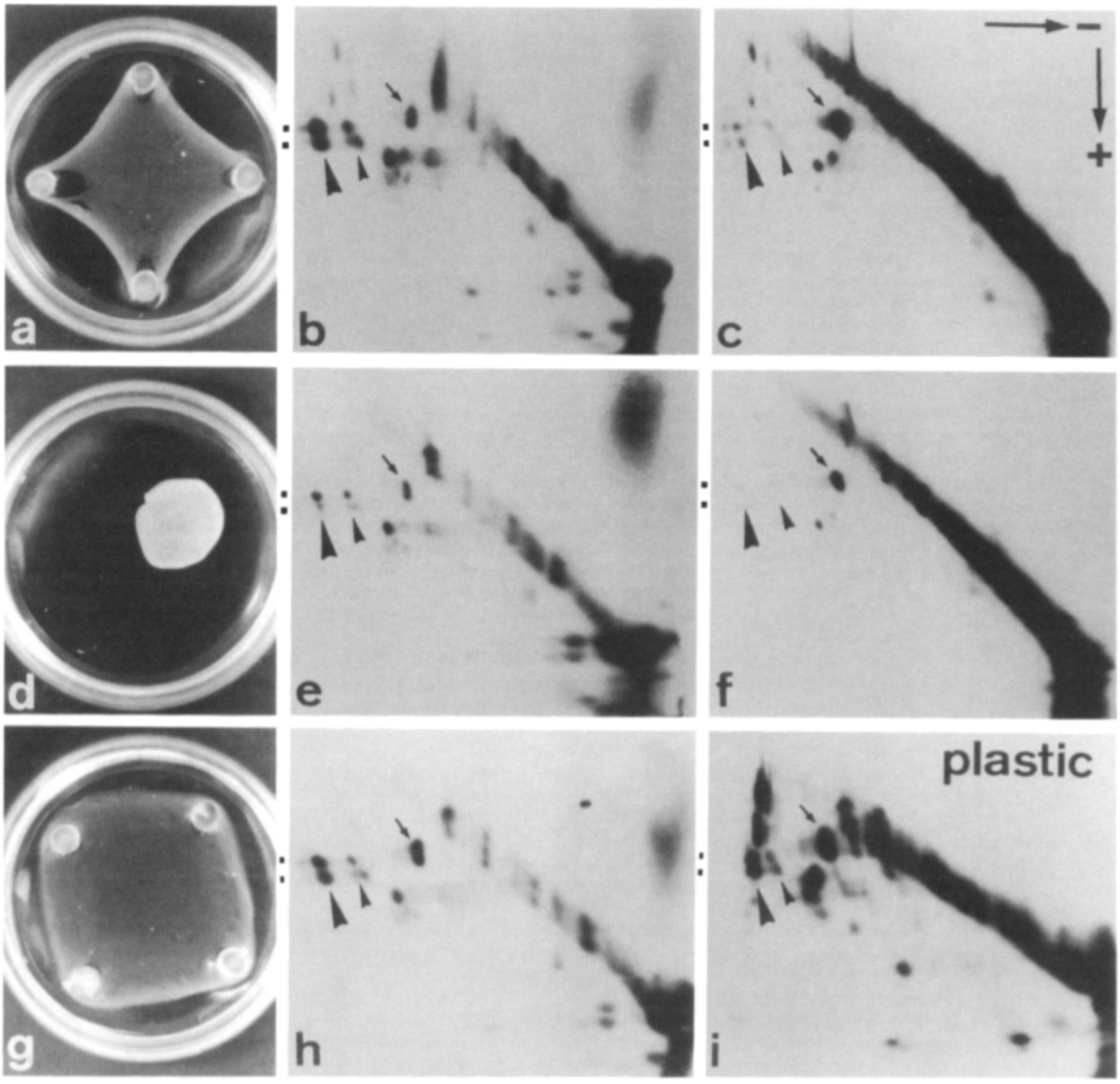


Figure 1. Extracellular matrix production in stressed versus contracted collagen gels. Chick embryo fibroblasts were cultured in a fixed (*a* and *g*) or a floating (*d*) collagen gel with the addition of cytochalasin B (*g*). The metabolically labeled medium of the stressed (*b*), the contracted (*e*), the cytochalasin B-treated culture (*h*), and a culture on plastic (*i*) as well as the cell extracts of the stressed (*c*) and the contracted (*f*) culture were analyzed by 2 d SDS-PAGE. The first dimension was run under nonreducing condition and the second dimension under reducing condition as indicated on the top right. The spots representing tenascin-C are marked by arrowheads. Both the tenascin-C hexamers (*large arrowheads*) and the trimers (*small arrowheads*) are resolved into large and small subunits reflecting the tenascin-C splicing variants migrating between 190 and 230 kD (positions marked by black dots to the left of each gel). The arrow points to the spot of fibronectin.

cin-C was found to be one of the most conspicuous proteins that showed a high expression under restrained conditions, whereas synthesis of other extracellular matrix proteins, such as fibronectin, was less affected. Since collagen gel contraction by fibroblasts depends on their functional cytoskeleton, it is inhibited by the actin-depolymerizing drug cytochalasin B. Indeed, in restrained, cytochalasin B-treated cultures, the collagen matrix remained in a relaxed configuration (Fig. 1 *g*). Interestingly, the cytochalasin B-treated culture (Fig. 1 *h*) showed reduced levels of tenascin-C synthesis when compared with the culture on the attached collagen gel, even though the shape of the collagen gel had remained in its original size. Thus, without a change in cell density, inhibiting the generation of tension by cytochalasin B correlated with a decreased level of tenascin-C secretion, while overall protein synthesis was not affected. On plastic in medium containing 5% FCS, the fibroblasts secreted a high amount of tenascin-C similar to the situation on the restrained collagen gel (Fig. 1 *i*).

The difference in tenascin-C accumulation in restrained (with the collagen gel attached to the culture dish) versus unrestrained collagen gels was confirmed by immunofluorescence studies. Culture wells photographed two days after plating of the cells are shown in Fig. 2 *A*, revealing that they have contracted the floating but not the attached gel considerably. Cryosections of both kinds of gels were stained by monoclonal anti-tenascin-C and anti-fibronectin antibodies, respectively, revealing the presence of fibronectin in both types of gels, while only the attached and thus stressed collagen gel contained detectable tenascin-C (Fig. 2, *B–E*). Using this experimental set up, accumulation and deposition of tenascin-C in the stressed collagen gel was observed in cultures held at 0.1% FCS, but not at 10% FCS, because high serum levels induced the expression of tenascin-C also in the floating, contracted collagen gels sufficiently to allow for staining of tenascin-C in the matrix by immunofluorescence (not shown).

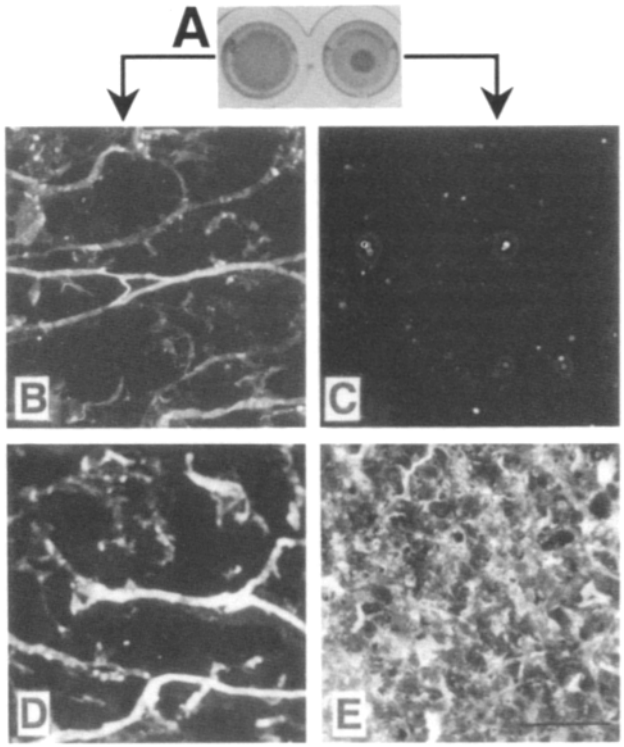


Figure 2. Cells deposit tenascin-C in stressed, but not in contracted collagen gels. Chick embryo fibroblasts were cultured within collagen gels in 96-well cluster plates for two days in medium containing 0.1% FCS. In A to the left a culture of an attached gel which fills the entire well is shown, and to the right a floating, contracted gel is visible. Stressed (*B* and *D*) and unstressed (*C* and *E*) gels were fixed, and cryosections thereof were stained with anti-tenascin-C (*B* and *C*) and with anti-fibronectin (*D* and *E*). Fibronectin was present in both kinds of gels, whereas tenascin-C was only detectable in the attached, stressed collagen gel. Bar, 200 μm.

To investigate whether induction of tenascin-C synthesis occurred at the level of transcription, we isolated the promoter region of the chick tenascin-C gene and used constructs of various length to drive luciferase expression upon transient transfection of chick embryo fibroblasts. The largest construct contained 4 kb of 5′ region of the transcription start site of the tenascin-C gene (p40Tn) and successively smaller constructs containing about 1200 bp (p12Tn), 600 bp (p06Tn), 200 bp (p02Tn), or no promoter sequence (p-pro) were prepared. The exact sequences contained in each of these tenascin-C promoter constructs can be seen in Fig. 6. The constructs were transfected into chick embryo fibroblast cultures and first compared for their activity in promoting luciferase expression upon induction with medium containing increasing FCS concentrations (Fig. 3). The results were compared to a construct containing the SV40 promoter (pSV40) as well as to the amount of endogenous tenascin-C protein secreted in the very same cultures. The results show that all constructs, except for the promoterless plasmid, were induced by FCS, but to a different extent. Thus p40Tn and p12Tn were equally active as pSV40 in promoting luciferase expression. p06Tn showed an intermediate activity and p02Tn was the least active promoter

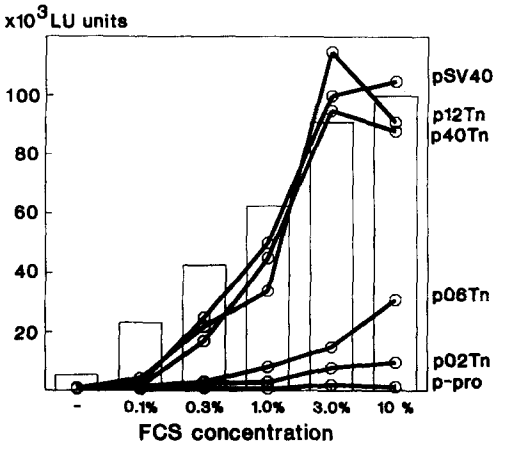


Figure 3. Serum response of TN promoter constructs. Chick embryo fibroblasts were transfected with promoter constructs containing luciferase as reporter gene. Cells were transfected with the constructs pSV40, p40Tn, p12Tn, p06Tn, p02Tn and with a promoterless construct p-pro, as indicated to the right of the graph. Each construct was analyzed for promoter activity at the FCS concentrations indicated. The bars behind the curves for luciferase activity (*LU units*) reflect the relative amounts of endogenous tenascin-C accumulating in the medium of the transfected cells as determined by ELISA assays as described previously (Pearson et al., 1988).

construct. The induction of the luciferase activity paralleled the increased accumulation of tenascin-C in the conditioned medium of these cultures. In a control experiment using cotransfection with a β-gal reporter plasmid we found that the transfection efficiency was equal with each of the different promoter constructs used. Therefore we can conclude that the 4 kb of 5′ sequences of the tenascin-C gene present in p40Tn encompasses very strong promoter activity, which is fully retained in the 1.2 kb p12Tn plasmid. Deleting the promoter region further results in a gradual loss of its activity. Using the same experimental set up, we tested the effect of TGF-β on the tenascin-C promoter constructs. Although we found an increase in the secretion of the endogenous tenascin-C protein in the transfected cultures, there was no detectable increase in the tenascin-C promoter driven luciferase expression for any of the constructs.

In our next set of experiments, we wanted to find out whether the tenascin-C promoter constructs could be induced by culturing the transfected cells on attached versus floating collagen gels. Because we feared that the transfection efficiency would be reduced if the cells were plated within the collagen gel, we decided to plate them on the surface of collagen gels for these experiments. We compared the results obtained from transfected cells cultured in 0.1% FCS under three conditions: on plastic, on attached collagen gels, and on floating collagen gels. The following cell morphologies were observed at the time of harvesting to make cell extracts for the determination of luciferase activity. The cells on plastic in low serum were very flat, whereas the cells in high serum were less spread out and more bipolar. The cells on the collagen gels (in 0.1% FCS) were very thin and elongated and in the case of the attached collagen gel had extremely long processes which were less prominent in cells on the floating gels, presumably because the gel had given way to the pulling by the cells. The floating gels were contracted to about one third of their initial volumes, but because of the much lower cell density, gel contraction was not as dramatic as in the experiment shown in Fig. 2. We determined luciferase activity under the different culture conditions after transfecting the cells with pSV40, p12Tn, p06Tn, or p02Tn, respectively. We compared the activity of the tenascin-C promoter constructs to pSV40 under each culture condition. The results presented in Fig. 4 demonstrate that certain tenascin-C promoter constructs show a large increase of activity relative to pSV40 in the cultures on the attached collagen gel, and a decrease on the floating collagen gels.

	Plastic	Attached collagen gel	Floating collagen gel
pSV40	100%	100%	100%
p12Tn	29%	62%	38%
p06Tn	15%	102%	17%
p02Tn	4%	7%	4%

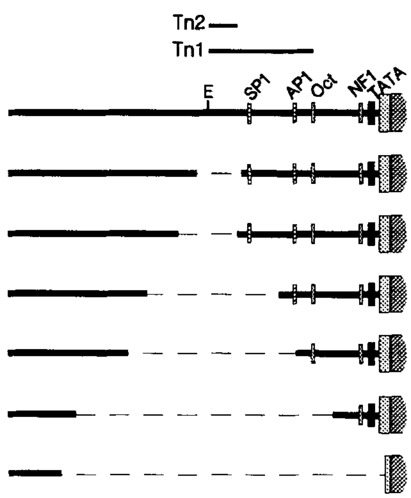
Figure 4. Stress-induced activity of the tenascin-C promoter. Cells cultured in 0.1% FCS either on plastic, or on attached versus floating collagen gels transfected with the tenascin-C promoter constructs p12Tn, p06Tn or p02Tn, respectively, were investigated for their promoter activity by measuring the activity of the reporter gene luciferase. The data were normalized to the luciferase units (LU units) obtained for pSV40 to correct for differences in transfection efficiencies between the cells on plastic versus the cells on the collagen gels. The actual data obtained for pSV40 and assigned as 100% were 1.83×10^6 LU units on plastic, 0.11×10^6 LU units on the attached and 0.50×10^6 LU units on the floating collagen gels, respectively.

This increase in activity on the stressed gels was retained by p06Tn, but not by p02Tn.

To narrow down the important regions of the tenascin-C promoter which are involved in the regulation by serum and by the collagen gel, a series of Bal31 deletion constructs were investigated. These new constructs are shown schematically in Fig. 5 and their precise sequences in Fig. 6. We used p12Tn as the starting construct, cleaved it at its single Eco47III site (which is the start point of p06Tn) and deleted increasing stretches of promoter sequences in both directions using BalI. We sequenced each of the constructs to exactly determine the deleted regions (Fig. 6). In Fig. 5, to the right of the schematic representation of the constructs, the results of the transfection experiments are shown. Interestingly, the response to serum and to a stressed collagen gel appeared to depend on completely different regions in the tenascin-C promoter.

The serum response was slightly decreased in p12/b2, showed a marked increase in p12/b3 (particularly noticeable at low serum concentration), and was entirely lost in p12/b4. We therefore speculate that in p12Tn/b3 either an inhibitory sequence has been lost, or two activating sequences present in the regions bordering the deleted area have been brought into a productive configuration. The deletion of the SP1 binding consensus sequence (Mitchell and Tjian, 1989) in p12Tn/b3 did not appear to have any negative effect on the tenascin-C promoter activity suggesting that this binding site may not be functionally important. After a further deletion in p12Tnb4, the response to serum was lost. In this construct, the first two nucleotides which are known to be important for the activity of the activator protein 1 (AP1) element (Risse et al., 1989) have been lost, suggesting that AP1 may be involved in the serum response. Of course, we cannot exclude the importance of other sequences deleted in this construct, which e.g., contain, next to the AP1-binding site, a consensus sequence for the binding of the transcription factor PU.1 (Klemsz et al., 1990). The presence or absence of the octamer sequence (Mitchell and Tjian, 1989) in the constructs p12Tn/b4 and b3 did not affect the activity of the constructs under our experimental conditions.

The increased response observed on a stressed collagen gel is immediately lost even in the shortest deletion constructs. We thus speculate that a region of the tenascin-C promoter crucial for the activity on the stressed collagen gel lies within 100 bp 3′ of the Eco47III site, the site where p06Tn starts. To further substantiate this finding we investigated whether we could transfer this postulated promoter element to a heterologous promoter, thereby rendering the hybrid promoter more active in cells on stressed collagen gels. Since we knew from the previous experiments described above that the SV40 promoter was only weakly active in cells on collagen gels, we used pSV40 as our test construct. We cloned the tenascin-C promoter regions labeled Tn1 and Tn2 in Fig. 5 in front of the SV40 promoter resulting in the plasmids pTn1-SV40 and pTn2-SV40. These constructs were tested under the same culture conditions used previously and compared to pSV40. The β-gal activity measured for the cotransfected pRSV-β-gal was equal with either type of SV40 promoter construct within one given culture condition. We therefore calculated the activity of the hybrid promoters relative to the original SV40 promoter under each of the culture conditions used. As can be seen in Fig. 7, the luciferase ac-



Construct	Plastic 10%FCS	Plastic 0.1%FCS	Attached collagen 0.1% FCS	Floating collagen 0.1% FCS
p12Tn	47	11	27	4
p12Tn/b1	41	17	4	8
p12Tn/b2	29	19	2	1
p12Tn/b3	41	52	8	2
p12Tn/b4	6	3	2	1
p12Tn/b5	4	8	1	1
p12Tn/b6	0.6	0.1	0.2	0.2

Figure 5. Localization of promoter regions responsible for the induction by FCS and by the stressed collagen gels. The left part of the figure shows a schematic representation of the deletion constructs p12T/b1-6 created from p12Tn by cutting it at the single Eco47III site (*E*) and digesting with Bal31 for various time periods before relegating the partially deleted promoter constructs. Some obvious potential sequences involved in promoter activity such as a consensus sequence for the binding of SP1, AP1, Octamer factor (*Oct*), NF1 and the TATA box are indicated. The exact sequences of all constructs are given in Fig. 6. The results for all constructs are presented as % luciferase activity measured for pSV40 under each of the culture conditions as described in the previous figure.

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┌ p12Tn
CATGGAGGCTGGGGAAGGCCAGCTGGGAGGAGGCACACTCAGAAAGGGAGAATGCTCAGAAGGGCCTGGACAGTCCCTCTTAATTCTTGTGAGCACCCTT
-1215
                                                                                         ├ b6
GTTGCTGAATAGGACCCCACTGCTTTCTTACTGGGCAGTCCCACAGCCTCCTGAGGTAGGTGGGTAATGCTTAAAGGGATTTGGATCAGTCCTGCTTCTG
-1115
                               ├ b5
AAATGGTGAAGCAATTTCATCCCTTCTCCTAGAACACAGACATGACAGAGCTTAACTCTCTAAGAGCTTAACCCTGCAGCTCCCCTCCCTCTAACTACCA
-1015                                GR
                                                                                                 ├ b4
GCCCCCCCCCACCCCCGAAAGAAAGGTAGATTCAGATAGAAGGGAGCTGACTGCTCCCTGAGTGCTAACTGTGATCACAATTCTTTGCCATGAGGCTGAG
-915
                                                              ├ b3
ATTTGAGGGTAAGCAGGAGACCCTCCCTCTGAGCAGCACTGTGCATTGCCCGCAGGTCCGGGACTGACCAAGGGACTCACGGAGGGGTTGGAGGATCTTT
-815
                                                               ├ b1
CCAACACCCAACTGCACAGTCTCTCTCTAAAAAGAAGGAAGCCCTCCTGTGCGCTTCTGTGGGCTTTTATCTCTCTCTTTTTAAGAGAATCAGCTTAGGA
-715
                ├ b2                                         ┌ p06Tn
TGCTGCTCAGCCCTGCTGTCCCCCGCAGCTGAGGGAGAGCAGAAGCAGCGCTGGGATGCAGAGATCACAAGCCTGGAAAGACCGATGTGTGCCAGATCAG
-615                                                      ←Δ→
                                                     b1┤        b2┤
CAGCTTGTGTACATCCGAGCTGCTCTGCTCATCCCTGCGGAGCAGTGCCAGCCCCACTCGCTGCCCATCCGCCCCAGCACACTCTGAGGACTTTTTGGA
-515                                                                   SP1
                                                                                       b3┤
ATGGAAACCCTGTGTTTGTGATCCCCCTGATTCCAGTTTGCTGCTCTTTCGCAGTGTTAAAATAAAGGGGAAAGGGGTTTAAAATTCCTGATAATGCACT
-415
                                      b4┤
CTGCTGATGCTGTAAGGAAAATATTCCTCTTTCTGAGTCATTTGCCCTGAGCTGACTTTTTTTTTTTTTGTTTGAAGGGTTCAGGGGTTTCCTTCTTTAG
-313                             PU        AP1
                ┌ p02Tn                                         b5┤
CCATAAACTGCATGCAAATTCTTTGTGTGTGTGTGTATGACAGTGTGTGTCTGCGAGTGCGTGTGTGTTTCTTCAGAAGCTAAGCCAAAGAGAGAGGCTG
-215             OCT

TTCCTTTAAAGCAAAACTGAAAAAAAAAAATGCCTTCAGGCATCCTATTACAGTGGAATGAGCCAAGGATCAGGATTTGCAGCTCAACTATATAAACCCGG
-115                                                                  NF1
                     +1              b6┤
CAGAATCTGGCTGATCTGACCAGTGTGCCGCACTGTCAAACCCTCCTTTCACACACGCG
-15
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Figure 6. Sequence of the tenascin-C promoter region and the various constructs. The entire sequence of p12Tn is given. It includes 1215 bp downstream of the transcription start site marked +1 and 44 bp of the first exon (printed in bold), which was joined to the luciferase gene of the reporter plasmid. The start points of p06Tn and p02Tn are marked. p06Tn starts at the Eco47III site used as starting point for the Bal31 deletion clones (←Δ→). The beginning and end of each of the Bal31 deletion clones is marked (b1–b6). The following consensus sequences are printed in bold, underlined, and marked: an AP1-binding site (*AP1*; Risse et al., 1989), an octamer sequence (*OCT*; Mitchell and Tjian, 1989), and a Nuclear Factor 1-binding site (*NF1*; Rossi et al., 1988). The TATA box is printed in bold and double underlined. Two other potential transcription factor binding sites for glucocorticoid receptors (*GR*; von der Ahe et al., 1985) and a consensus sequence for the binding of PU.1 (*PU*; Klemsz et al., 1990), respectively, are underlined and labeled.

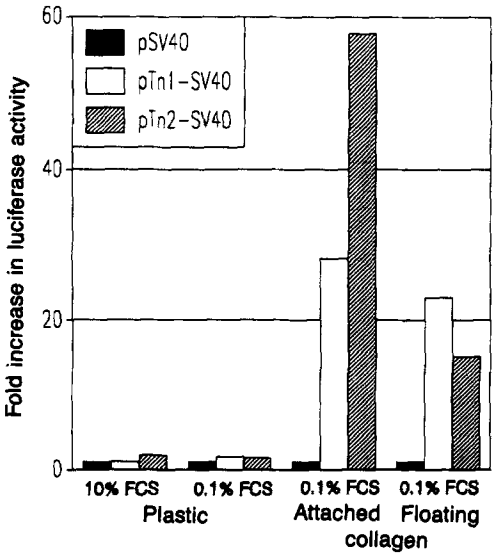

Figure 7. Transfer of the "collagen response element" to a heterologous promoter. The tenascin-C promoter segments marked Tn1 and Tn2 in Fig. 5 were cloned in front of the SV40 promoter of pSV40 resulting in the plasmids pTn1-SV40 (addition of the sequence from −570 to −202) and pTn2-SV40 (addition of the sequence from −570 to −469). The hybrid promoter constructs were transfected together with pRSV-β-gal (for normalization) under the conditions marked in the figure. The luciferase activity measured in the cell extracts is shown relative to the activity measured for pSV40, which arbitrarily was set to 1. Clearly the addition of the tenascin-C promoter segments to the SV40 promoter dramatically increased its activity in cells cultured on attached collagen gels and this increase was reduced on floating gels.

tivity measured in cells on plastic was not significantly different between the three constructs, however, on the stressed collagen gel we noted a 30- to 60-fold increase in activity of the tenascin-C-SV40 hybrid promoters and this increase was reduced on floating collagen gels. The transfer of the 102 bp identified in the deletion constructs to contain the necessary sequences for the increased promoter activity in cells on stressed collagen gels (pTn2-SV40) was sufficient to activate this reporter construct on cells cultured on stressed collagen gels.

Discussion

Since tenascin-C is an extracellular matrix protein with rapidly changing expression patterns during embryogenesis as well as during regenerative processes, it is interesting to study the mechanisms regulating its expression. It is known that serum and certain growth factors can induce the accumulation of tenascin-C mRNA and protein (see Introduction). One of these factors is TGF-β (Pearson et al., 1988), which is known to induce the synthesis of many extracellular matrix proteins as well as their cellular receptors (Massagué, 1990). In the case of mouse α2(I) collagen, promoter constructs have been used to define the promoter element responsible for TGF-β induction. In this case a nuclear factor 1 (NF1)-binding site conferred the TGF-β responsiveness to the reporter construct (Rossi et al., 1988). Another example is TGF-β1 itself which can up-regulate its own expression (Van Obberghen-Schilling et al., 1988), apparently requiring AP1 (Kim et al., 1989). The tenascin-C promoter constructs used in our present studies contained both consensus sequences for the binding of both NF1 and AP1. Nevertheless, we did not detect any stimulation by TGF-β of the reporter gene after transfection of chick embryo fibroblasts. However, in the same cultures we observed an increase of endogenous tenascin-C production. It appears therefore that sequences other than the 4 kb of the tenascin-C promoter are required for the stimulation of tenascin-C transcription by TGF-β. Alternatively, the induction of tenascin-C mRNA by TGF-β is due to an increase in the mRNA half-life.

Although the AP1-binding site was not able to provide responsiveness to TGF-β, it may be important for the regulation of tenascin-C expression by serum, since its deletion correlated with the loss of serum induction. Serum activates transcription from p12Tn more strongly than from p06Tn, therefore we can postulate the existence of further control elements in the region between −567 and −1215. Since the construct p12Tn/b4 drops dramatically in inducibility, it could be that not only the loss of the AP1 site is responsible but that important regulatory sequences are contained in the region between −766 and −829. In the construct p12Tn/b3 that showed increased responsiveness to serum, this segment was brought closer to the transcription start site while it was lost concomitantly with the AP1 site in construct p12Tn/b4 resulting in very poor activity. The sequence of this promoter segment, however, does not contain any sequence elements reported to be binding sites of known transcription factors. We have no evidence for an involvement of the SP1 or octamer sequences in the activation of the tenascin-C promoter. We are also not able to speculate about the functionality of the NF1 site, since the construct in which this region is deleted has also lost the TATA box and is completely inactivated. Recently, it was reported that tenascin-C expression can be down-regulated by glucocorticoids (Ekblom et al., 1993). In this context it may be interesting that a consensus sequence for the binding of glucocorticoid and progesterone receptors (von der Ahe et al., 1985) is located at position −985 of the tenascin-C promoter sequence.

In addition to the induction of tenascin-C by serum or growth factors, we have shown that tenascin-C expression is stimulated when cells are cultured in attached collagen gels, but repressed in floating collagen gels. This is not simply due to the general reduction of protein synthesis, which has been shown to occur in contracting gels (Mochitate et al., 1993). Instead, tenascin-C is preferentially affected when compared to other proteins. This difference in tenascin-C synthesis is more clearly seen when the cells are cultured in low serum, since serum by itself stimulates the synthesis of tenascin-C even in contracted collagen gels. Interestingly, reporter constructs under the control of the tenascin-C promoter were also subject to regulation by the state of the collagen gel, suggesting that the regulation of tenascin-C synthesis occurs on the level of transcription.

What could be the reason for differential production of tenascin-C on attached versus floating collagen gels? One possible explanation is that the state of the collagen gel affects tenascin-C synthesis indirectly. Altered cell–cell contact, for example, could influence the release of autocrine or paracrine factors, which in turn might change the level of tenascin-C synthesis. Alternatively, the effect could be mediated more directly through the mechanical traction which fibroblasts exert on a fixed collagen gel, generating strain which cannot be relieved by gel contraction. This strain

might induce the synthesis of growth factors (Wilson et al., 1993) which in turn induce tenascin-C, or tenascin-C itself is induced by strain, whereas cells in a contracted, relaxed gel synthesize less tenascin-C. Mechanical stress affects the organization of the cytoskeleton and thus cell shape, and cell shape changes can affect gene transcription and differentiation of cells (Ben-Ze'ev, 1991). Since cell shape largely depends on the interaction of cells with the surrounding extracellular matrix, it is not surprising that extracellular matrix can have decisive functions in regulating gene expression, differentiation and development (Bissell et al., 1982; Adams and Watt, 1993; Juliano and Haskill, 1993). The signal transduction mechanism from the extracellular matrix to the cytoskeleton is apparently mediated by integrins and can lead to the activation of second messenger pathways similar to those involved in growth factor stimulation of cells (Hynes, 1992; Schwartz, 1992; Zachary and Rozengurt, 1992). Furthermore, "mechanochemical transduction" is mediated by integrins, which transmit mechanical signals to the cytoskeleton (Wang et al., 1993). Fibroblasts within a collagen gel have been shown to use collagen receptors of the α1 integrin class for adhesion to and the contraction of the gel (Gullberg et al., 1990). Specifically, $\alpha 2\beta 1$ seems to be involved (Klein et al., 1991). Interestingly, the cytoplasmic tail of the integrin α chain is important for this function. Cells transfected with plasmids encoding the α2 extracellular domain linked to its own as well as to the α5 cytoplasmic tail, but not to the α4 cytoplasmic tail, were able to contract collagen gels. Cell adhesion was equal in all cases and cell migration activity was highest in the cells containing the chimera with the α4 cytoplasmic tail (Chan et al., 1992). These experiments indicate that occupation of different types of integrins can have distinct intracellular effects.

Important new findings relating to integrin-mediated signal transduction came from the studies of monocytes (for review see Juliano and Haskill, 1993). These cells represent a good model system, since adherence to extracellular matrix dramatically changes the phenotype of these otherwise nonadherent, circulating cells. Integrins were shown to be a primary signal transduction molecule in this event (Yurochko et al., 1992). A number of adherence-induced cDNAs have been cloned, some of which are transcription factors and cytokines. Interestingly, the nature of the substratum and the type of extracellular matrix protein encountered by the monocytes elicits differential responses (Sporn et al., 1990). Obviously, cytoskeleton associated transcription regulators, such as e.g., the LIM domain containing proteins zyxin and CRP (Sadler et al., 1992) or IκB (Lux et al., 1990; Haskill et al., 1991) are candidate molecules for mediating adhesion-stimulated changes in gene expression. Furthermore, cytoskeleton-bound kinases and phosphatases could be involved in cytoskeleton-dependent activation processes of cytoplasmically located transcription factors, the function of which are often influenced by their phosphorylation status. We hypothesize that in the experiments described in this paper the tenascin-C gene transcription on stressed collagen gels could be influenced by these types of second messenger proteins and transcription factors.

The question whether there are specific promoter regions such as "extracellular matrix-response elements" is at the moment still open (Adams and Watt, 1993). Only a few cases have been described where extracellular matrix or mechanical stress induces the transcription of specific genes. Thus in cardiac myocytes stretching of the deformable culture dishes induces the transcription of c-fos and α-actin (Komuro et al., 1991). This response could also be seen after transfecting the cultures with reporter constructs under the control of the c-fos promoter. The responsible promoter element appeared to be a serum response element under the control of the protein kinase C second messenger pathway (Komuro et al., 1991). The application of fluid shear stress to endothelial cells influences the expression of many genes, among them platelet-derived growth factor-B (PDGF-B). It was possible to identify a *cis*-acting element in the PDGF-B promoter conferring shear stress induction to a reporter construct (Resnick et al., 1993). The core sequence of this element was GAGACC. The same sequence is also found in many other promoters of shear responsive genes. In the tenascin-C promoter such a sequence is not present, but within the 100 bp identified here to be involved in activation of the tenascin-C gene in cells on stressed collagen gels, a related sequence with only one mismatch was found twice. In the case of albumin transcription in hepatocytes, culturing of the cells on collagen gels has been shown to induce transcription of factors that bind to a liver-specific enhancer element (Liu et al., 1991). Furthermore, a region in the β-casein promoter could be identified that led to extracellular matrix induced gene expression by mammary cells. However, the responsive region of the promoter could not be separated yet from the region mediating prolactin-induced transcription (Schmidhauser et al., 1992). Clearly many more genes and promoters have to be analyzed, the response elements have to be exactly identified and the transcription factors characterized, before any definitive claims can be made. Our own results provide further hints towards the potential existence of an "extracellular matrix response element," since within the tenascin-C promoter we could clearly distinguish the promoter region involved in serum induction from the one mediating the response to a stressed collagen gel.

To affect transcription by mechanical forces is an interesting possibility for regulating gene expression during development. A review addressing this question has recently been published (Ingber, 1994). It is conceivable that during epithelial growth in organogenesis, stress is exerted on cells in the surrounding tissue. This is exactly the location of increased tenascin-C expression in developing organs such as the mammary gland, feather buds or teeth (Chiquet-Ehrismann et al., 1986). Perhaps the epithelium induces tenascin-C expression not only by the release of growth factors, but also through the generation of mechanical forces acting on the surrounding mesenchymal cells. Another example might be the generation of bone spicules which develop along the lines of forces acting on the bone. Tenascin-C is also present during bone formation and one could consider a possible stress-related expression. Further examples for physiologically relevant stress responses could be the expression of tenascin-C in wounds during wound contraction, or the expression of tenascin-C around tumors (see Chiquet-Ehrismann, 1993 and references therein). It will be interesting to further investigate whether indeed mechanical stress can mediate the effects on tenascin-C gene expression, to delineate the mechanism of action, and to determine the potential physiological importance of such a regulation for tenascin-C and other proteins.

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