Identification of an enhancer involved in tissue-specific regulation of the rat fibronectin gene

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ABSTRACT

Fibronectin (FN) is a widely distributed extracellular matrix protein that is essential for cell adhesion in a variety of biological processes such as wound healing, tissue development and remodeling and oncogenic transformation. Appropriate FN levels are obtained by induction or repression of the FN gene in response to specific factors or circumstances *in vivo*. In order to identify regulatory regions involved in tissue-specific expression of FN, we have examined the transcriptional activity of overlapping fragments, within 4 kb upstream of the rat FN gene, following transfection into different cell types. Two regions conferred increases in transcription. The region between –1.08 and –2.6 displayed tissue-specificity and was active in fibroblasts but not hepatoma cells. The second region, between –3.2 and –3.9, was active in both cell types. Further characterization of the –1.08 to –2.6 segment demonstrated that it acts as an enhancer. Exonuclease III deletions of the 3' and 5' ends of the enhancer localized essential sequences between –1.5 and –1.7 and indicate that this fragment acts in concert with other sites between –1.08 and –2.6 to provide maximum enhancer activity. Gel mobility shift assays demonstrated fibroblast-specific binding of nuclear protein(s) to a 65 bp fragment within the essential region and DNase I footprinting localized this binding to a 27 bp sequence. Deletion of the sequence abolished the activity of the 1.5 kb enhancer. These studies show that a novel DNA sequence at –1688 is involved in regulating transcription of the FN gene in fibroblasts.

INTRODUCTION

Fibronectin (FN) is a large structurally and functionally diverse glycoprotein that is produced by many different cell types (1,2). Two major FN isoforms, cellular FN and plasma FN, are found *in vivo* and are produced by fibroblasts and liver, respectively. Cellular FN is incorporated into the tissue extracellular matrix, while plasma FN is a major blood protein. Tissue-specific expression of these FN isoforms is regulated at both the transcriptional and post-transcriptional levels. Post-transcriptional modifications occur via alternative splicing (3–5). Transcriptional

control plays an equally important role in modulating FN levels in specific tissues and in response to certain stimuli (6). Expression of cellular FN is regulated in numerous biological processes where modulation of cell adhesion occurs such as during cell migration, development, matrix formation and wound healing (1,2). On the other hand, plasma FN levels are kept fairly constant in the blood except under conditions of severe tissue injury and inflammation, when acute phase reactants (of which FN is one) are produced by hepatocytes (1).

FN gene transcription is induced by serum (7,8), epidermal growth factor, platelet-derived growth factor, transforming growth factor- β (7,9,10), cAMP (9) and gamma-interferon (11). In addition, oncogenic transformation of fibroblasts causes reduced production of FN due to decreased transcription (12). The FN promoter contains basal transcription elements as well as specific regulatory consensus sequences (13,14). A G10 stretch and two GC boxes have been shown to be E1A-responsive elements and appear to be involved in repression of rat FN gene expression (15). Three different cAMP response elements (CRE) have been described in the human FN promoter. One high affinity and two low affinity CREs mediate the cAMP response through the binding of three proteins, PEA2, CREB and an unidentified factor (16). A CRE was also shown to suppress FN expression in granulosa cells (17). In addition, CRE and CCAAT-binding proteins play interactive roles in both the human (18) and rat (19) FN genes.

The above mentioned elements are located within the first 500 bp 5' of the FN gene. As FN functional diversity necessitates additional regulatory mechanisms, we are interested in identifying upstream regions involved in the tissue-specific regulation of the rat FN gene. In the present study, a series of 5' fragments ranging to –3.9 kb were cloned upstream of the chloramphenicol acetyltransferase (CAT) reporter gene and examined for transcriptional regulatory activity. We demonstrate that two regions exhibit positive effects on transcription. The region between –1.08 and –2.6 was active in fibroblast but not hepatoma cells while the –3.2 to –3.9 segment was active in both cell types. The 1.5 kb fragment between –1.08 and –2.6 acts as a characteristic enhancer element in fibroblasts and we have localized an essential region to the area between –1.5 and –1.7. In addition, mobility shift assays using a 65 bp fragment and fibroblast or liver extracts show distinct patterns of nuclear protein binding and DNase I footprint analysis localized the binding region to a 27 bp site. Deletion of most of this sequence from the 1.5 kb fragment results in the loss of enhancer activity.

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Figure 1. Diagram of overlapping FN promoter-CAT constructs. A series of FN promoter fragments was derived from a rat FN genomic clone. The *Pst*I site at +0.137 forms the 3' ends of all the fragments. The 5' ends are bounded by the indicated restriction sites and their positions are shown in kilobases relative to the transcription start site (+1).

MATERIALS AND METHODS

FN promoter constructs

All constructs were made using a pGEM-2 plasmid backbone (Promega). Following the addition of *Hind*III linkers, the CAT gene was cloned into the *Hind*III polylinker site of pGEM-2. This construct, pCAT, was then used as a negative control and for construction of FN promoter-CAT plasmids. The overlapping FN promoter fragments, derived from a 15 kb rat genomic clone (14), extended from a *Pst*I site at position +137 in the 5' untranslated region. The 5' ends of the fragments were chosen using convenient restriction sites between -0.32 and -3.9 (Fig. 1). For use as a positive control, the Rous sarcoma virus long terminal repeat (RSV LTR) attached to the CAT gene was isolated from the vector pRSVcat (20) and ligated into pGEM-2. This vector will be referred to as pRSVCAT. To construct an RSV-lacZ plasmid, pRSVCAT was digested with *Hind*III to remove the CAT sequence and the *lacZ* gene from pCH110 (Pharmacia) was inserted in its place. For the construction of p1.5FNCAT and p1.5(-)FNCAT, the 1.5 kb fragment from -1.08 to -2.6 was made blunt, ligated into the *Sma*I site in pFNCAT and isolated in both orientations. To construct p1.5TKCAT and p1.5(-)TKCAT, the same blunt-ended 1.5 kb fragment was cloned into a blunted *Xba*I site in pGEM-2 in both orientations. These plasmids were then digested with *Hind*III and *Bam*HI to excise the 1.5 kb fragments which were then ligated into *Hind*III and *Bam*HI digested pBLCAT2. pBLCAT2 (referred to here as pTKCAT) contains the Herpes simplex virus thymidine kinase (TK) promoter upstream of the CAT gene (21). The 1.5 kb fragment was sequenced by the dideoxy chain termination method (22) using Sequenase (US Biochemical).

Deletions in the 1.5 kb enhancer

The 1.5 kb fragment from -1.08 to -2.6 was cloned in both orientations into the *Eco*RI site of the Bluescript KS+ vector (Stratagene). The positive orientation was used to produce successive deletions from the 3' end of the 1.5 kb fragment and the negative orientation was used for deletions from the 5' end. Plasmids were linearized by digestion with *Kpn*I, which produced

a 3'-protruding end resistant to exonuclease III cleavage and with *Eco*RV which left the enhancer fragment susceptible. Deletions were made by digestion with 20 U exonuclease III (New England Biolabs) per μ g DNA at 30°C. Aliquots were removed at 1–2 min intervals, terminated with mung bean nuclease buffer and incubated with 4 U mung bean nuclease (New England Biolabs) per μ g DNA at 30°C for 30 min. Subsequent treatment with 3 U Klenow (New England Biolabs) per μ g DNA for 45 min at 20°C ensured complete blunting of DNA ends. *Hind*III linkers were added prior to religation of deleted plasmids.

Two internal deletions were made within the 1.5 kb enhancer. The plasmid p Δ 165FNCAT was constructed from p1.5 Δ 5FNCAT and p1.7 Δ 3FNCAT exonuclease III deletions. Fragments spanning -1572 to -1080 and -2547 to -1738 were ligated resulting in the 1.5 kb enhancer fragment with a 165 bp deletion from -1737 to -1573. For construction of the plasmid p Δ 33FNCAT, the 5' end of the 1.5 kb enhancer (from -1699 to -2547) was prepared using PCR amplification. The product was then phosphorylated, made blunt with the Klenow fragment of DNA polymerase and ligated into p1.5FNCAT previously digested to remove the sequence between -2547 and -1666. This resulted in a plasmid containing the 1.5 kb fragment with a 33 bp deletion from -1698 to -1666.

Cell culture and DNA transfections

NIH 3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% calf serum (Hyclone). HepG2, a human hepatoma cell line kindly provided by Shirley M. Tilghman (Princeton University), was maintained in Ham F12 nutrient mixture and DMEM at a 1:1 ratio containing 10% fetal calf serum and 10 μ g/ml of bovine pancreas insulin (Sigma Chemical Co.). The Rat1 cell line (F2408) was cultured in DMEM supplemented with 5% calf serum.

Cells were transfected using the GIBCO BRL calcium phosphate transfection system. Confluent 3T3 cells were split 1:20 1 day prior to transfection and HepG2 cells were split 1:5 3 days before transfection. Cells were transfected with equimolar amounts of plasmid DNA (between 10 and 23 μ g) supplemented with carrier DNA to give equal mass. HepG2 cells were cotransfected with an RSV-lacZ plasmid, the Rous sarcoma virus long terminal repeat fused to the bacterial *lacZ* gene, as an internal control for transfection efficiency. β -galactosidase assays were performed using cytoplasmic extracts as described in Sambrook *et al.* (23).

Transfection efficiency in fibroblasts was measured by dot blot analysis to quantitate plasmid DNA. Pelleted nuclei were lysed in 0.5 M NaOH, neutralized with 10 M ammonium acetate and diluted with a solution of 1 M ammonium acetate, 0.02 M NaOH for vacuum blotting onto nylon membranes (Micron Separations Inc.). As a control, known concentrations of a CAT plasmid were also immobilized. Membranes were then hybridized with a ³²P-labelled CAT DNA probe. Washed filters were used to expose a phosphor storage screen and hybridization was quantitated by analysis on a Molecular Dynamics PhosphorImager. All values were normalized to total protein in each extract and compared to the CAT plasmid control.

CAT assays

Cells were harvested 46–48 h after transfection, lysed in 0.25 M Tris HCl, pH 7.8, by three successive freeze/thaw cycles and centrifuged to pellet nuclei. Pellets were used for DNA dot blots

Figure 2. CAT activities in 3T3 cell extracts. Cells were transfected with the indicated FN promoter-CAT plasmids and cell extracts were assayed for CAT activity. Activity is expressed as pmol [^{14}C]chloramphenicol butyrylated per min per mg protein. Each column represents the mean value for an average of five experiments (minimum of two). The error bars represent standard deviations. A pCAT promoterless negative control and pFNCAT basal FN promoter are also shown.

as described above. Extracts were heated to 65°C for 7 min, to destroy possible inhibitory/deacetylase activity and stored at -20°C for use in CAT assays. A portion of HepG2 extracts was removed prior to heating for use in β -galactosidase assays. Protein concentrations were measured using the Bio-Rad protein assay. Either 1 or 25 μg of protein was used in CAT assays, dependent upon the level of activity of transfected constructs for a given cell-type. Reactions were carried out at 37°C for 30 min using 4 mM *n*-butyryl CoA (Sigma Chemical Co.) as the acyl donor and 0.05 μCi [^{14}C]chloramphenicol (NEN Research Products) as the substrate. CAT activity was measured by extraction of acetylated product with a 4:1 mixture of pristane:xylene (Aldrich Chemical Co., Inc.) and quantitation of the product by scintillation counting (24). pmol butyrylated were determined using the specific activity of the [^{14}C]chloramphenicol. Thin layer chromatography assays (20) were also performed on several samples and gave comparable results.

Gel mobility shift assays

Nuclear extracts were prepared from cell lines as described by Müller *et al.* (25) except 0.5% NP-40 was used for cell lysis instead of Triton X-100. A frozen crude nuclear pellet prepared from rat livers (26) was kindly provided by Elizabeth Sztul (Princeton University). The volume of the resuspended nuclear pellet was adjusted to 20 ml per liver with the nuclear buffer described by Wu (27). Nuclei were isolated by the method of Wu (27) with the following exceptions. Nuclear suspension was filtered through gauze instead of nylon after homogenization and before centrifugation through sucrose. Extracts were prepared from the rat liver nuclei using the NP-40 buffer mentioned above.

DNA probes were labelled with [^{32}P] dATP using Klenow fragment. Binding reactions were carried out in 12–15 μl total volume with 2 μg poly [dI/dC], 5 μg nuclear extract and 14 fmol

Figure 3. CAT activities in HepG2 cell extracts. Cells were transfected with the FN promoter-CAT plasmids plus pRSVlacZ. Each column represents the average of four transfections. The error bars represent standard deviations. All samples were normalized for transfection efficiency using the β -galactosidase assay as an internal control.

of ^{32}P -labelled DNA probe for 30 min at room temperature. Fifty-fold molar excess unlabelled DNA was added in competition assays. Reaction buffer contained 12 mM HEPES (pH 7.9), 10% glycerol, 5 mM MgCl_2 , 60 mM KCl, 1 mM DTT, 50 $\mu\text{g}/\text{ml}$ BSA, 0.5 mM EDTA and 0.05% NP-40. Bound and unbound DNA were separated in a 4% polyacrylamide gel (29:1, acrylamide:bisacrylamide) in $0.25\times$ TBE and gels were dried on DE81 paper (Whatman International Ltd.) and exposed to film.

DNase I footprinting

DNase I footprinting (28) was performed as follows. Nuclear extracts were prepared as described above. A 346 bp fragment from -1538 to -1883 used as the DNA probe was end-labelled with [γ - ^{32}P]ATP using T4 polynucleotide kinase. Binding reactions were carried out in 50 μl total volume using the reaction buffer described above plus 2 μg poly [dI/dC], 25 or 50 μg nuclear extract and 10 000 c.p.m. of labelled DNA probe for 30 min at room temperature. Reactions were treated with DNase I for 60 s at room temperature (amounts of DNase I used were determined empirically) and stopped by addition of 50 μl DNase stop solution (0.6 M sodium acetate, 20 mM Tris, pH 8.0, 1% SDS, 10 mM EDTA, 100 $\mu\text{g}/\text{ml}$ yeast RNA). Samples were then extracted with phenol/chloroform, ethanol precipitated and resuspended in sample loading buffer (80% formamide, $0.5\times$ TBE, 0.02% xylene cyanol, 0.02% bromophenol blue). Samples were analyzed on a 6% denaturing polyacrylamide gel, dried onto DE81 paper and visualized by autoradiography. A>C and G/A reactions were performed by rapid chemical sequencing of the end-labelled probe (29).

RESULTS

Analysis of fibronectin promoter activity in fibroblasts

In order to determine whether regions upstream of the FN gene contain tissue-specific regulatory elements, an overlapping series

Figure 4. Characterization of enhancer activity of the 1.5 kb fragment. Constructs containing the 1.5 kb fragment in both orientations immediately upstream of either the FN or TK promoter were transfected into 3T3 cells. CAT activity is shown relative to pFNCAT or pTKCAT. The dashed lines represent sequence that is not present in the constructs. The average number of experiments per plasmid was five except for p1.5FNCAT which is the mean of 11 experiments in this and subsequent figures. Values were normalized for transfection efficiency by dot blot hybridization as described in Materials and Methods.

of rat FN promoter fragments was generated (Fig. 1). These fragments, originating at +137 bp within the 5' untranslated region and ranging to -3.9 kb at the 5' end, were cloned upstream of the CAT reporter gene in a pGEM-2 vector as described in Materials and Methods. pFNCAT, which extends to -0.32 at the 5' end, contains the basal FN promoter including a TATAA box, two CCAAT boxes and two SP-1 sites (14). This plasmid was used to determine basal levels of FN transcription. Since fibroblasts are a major source of cellular FN, the plasmids were first transfected into NIH 3T3 cells and tested for activity using a [14 C]chloramphenicol phase extraction CAT assay (24). In these and all subsequent experiments, the promoterless plasmid, pCAT, served as a negative control and pRSVCAT, containing the RSV LTR, served as a positive control.

Comparison of CAT activity obtained with overlapping fragments after transfection into 3T3 cells showed an 8-fold increase with inclusion of the segment between -1.08 and -2.1 (Fig. 2). An additional 5-fold increase in activity was obtained with the segment between -3.2 and -3.9. Similar increases were also seen when these plasmids were transfected into primary rat embryo fibroblasts. These data identify a regulatory element that can enhance transcription in fibroblasts and lies between -1.08 and -2.6 kb upstream of the FN gene. No activity greater than that of the promoterless CAT plasmid was seen with any of the FN sequences when transfected into the pituitary cell line AtT20, which does not express FN, showing that the activity is specific to cells that produce FN.

Analysis of FN promoter activity in hepatoma cells

The human hepatoma cell line, HepG2, expresses a number of liver-specific proteins including albumin, transferrin and fibri-

Figure 5. CAT activities of exonuclease III deletion constructs. A series of deletions was made from the 5' end (A) and 3' end (B) of the 1.5 kb enhancer region and enhancer activity was measured after transfection into 3T3 cells. Activity was normalized to pFNCAT (value = 1.0). The average number of experiments per construct was four. Values were normalized for transfection efficiency by dot blot hybridization.

nogen (30) and has been used extensively to characterize liver-specific gene regulation (31,32). As liver is the source of plasma FN *in vivo*, these cells were used to examine the tissue specificity of the regulatory regions identified in fibroblasts. By comparison to the pCAT control, the basal promoter alone showed similar expression levels in HepG2 cells to that in fibroblasts, but addition of sequences between -0.32 and -1.08 resulted in a 3-fold decrease in activity (Fig. 3). Neither p-2.1CAT nor p-2.6CAT was able to enhance transcriptional activity in HepG2 cells. Activity with all size fragments between -1.08 and -3.2 was less than the basal promoter alone. This shows that the -1.08 to -2.6 region is not active in these cells. In contrast, a positive regulatory element between -3.2 and -3.9 was active in liver cells and was able to overcome the apparent repression of downstream sequences. Therefore, fibroblasts and liver cells have both common and distinct sites for regulation of FN expression.

Enhancer activity of the fragment between -1.08 and -2.6

To determine if the 1.5 kb fragment between -1.08 and -2.6 exhibits the characteristics of an enhancer element, this fragment was placed immediately upstream of the basal FN promoter as well as the heterologous TK promoter in both the positive and negative orientations. These plasmids were subsequently transfected into 3T3 cells and cell extracts were assayed for CAT activity. Relative to the pFNCAT basal promoter plasmid, p1.5FNCAT and p1.5(-)FNCAT increased transcription to the levels of p-2.1CAT and p-2.6CAT (Fig. 4). Therefore, the activity is independent of orientation. Similar increases were also seen in the context of the TK promoter in both orientations. These data

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-2547 AGTACTACAA ACAAAGGGC CAAACAGACA AACAAAACAA CCCTCCCAAA ACAAAACAG CCGACAACAG CAACAACAC AAAAACACCA AAACAACCC
-2447 CCAAAAGTTT GGTGAGATAT AACTTCGACT TATTCCCTAG AATTAGAGTT ACAGATGCTT TGTGAGTGGT CATGATCATG TGGTTGCTGG GAATAGAACC
-2347 CAGGTCTCTT GGAAGAGCAG CCTGTGCTCA TAACCATTGA GCTGTCTCTC CAGTGCCCTG ACCCTCAACT GTAGTCTTTA AGATTTAATT ATCTCTGAGC
-2247 TAGAGAGAAAT CGACTTGGAA TTGGAAGTGA AATGGATGTG GAAGATACT GTAGCAAGGC TGGTTTACCT TAATTAAGAA AATGCTCTCA TGTCCAAAT
-2147 CAAACCAAAA ATACAGCTTT CAAGTAAAG CATTCTATA AAATTCTGCC AAATATTTTT GCTTGTGGGC CACAGGAGAT ATTTTCTATG GGGCTGAAGG
-2047 AGAAAACCTT CTCTGAAAT GTAAAGTGGG TTCCCCGCAA AGTCTGTAA TAAGTATATT ATTCTAAAGT ATTTTGAGTA TGGTAAATAT ACTGAACAT
-1947 CATGTTTTTG TGACAAATTT CTTTCTAAC CGCTTATTC CTCTCTCAG GATCTGCCAT ATTTGTGGAA AGCCGTCCTC TCAGGTTTAT CTAGCAAGCA
-1847 TCCTGATTC CAGGTATCCT TCTCTCCCT GGAGAGAGCA CTCTCCAGAA CTCACTGTT TTACAAGGAG CCCTTTTATA TCTGAAAGAG AAAACGGTT
-1747 TGTCAAGGGA TGATGAGAAA TTCAAAAAA CTAAGTCAGA CGGGATTCTA AATTGGGGG AATGTATGGC ATTCGTTCAA CCCCGATTGT TGGTCACAAA
-1647 GATTCCTAC TTAATTTACA CCGCTGTAA GAACTGGTA TTCGTGTGG GTTCTCTGT TTTAGTCTT TTACATCAAT AAAAATAAAT GITAATTTTG
-1547 CTCTTTTAA GTGTGAGGA TTTTATAAG CATACAAATT CAAATTATCC TTACAGCGGA ACTTTTGCGC AAAACAAGAA CCTGTGACAA ATAACATATT
-1447 TTGCTGGTAT CTTAATGTG TTCTCAAAGC AAATGAGACC ACGTGCTAA CCGAAGGAGA AAGCCAAGT TGGAGTTCAG ACCTGCTGCT TTGAAAATAG
-1347 TGGCTTCTC ATTTTGAAG ACAGCAACTG GGCTGTGTGT GTCTGAAAGG TAAAAAAGAA AGAAAAAGAA AAAAAAGAA AGACCAACGT
-1247 GTTAGAGCTG TGAGCTTCAG GGGACGTCTC CTATCCCCGC TCCAATCTCA GTAGTGTAGA AAGTCCGGAG AATTTCCGGA GACTTTTCAG TGGAACTTTA
-1147 TACATTCTTG GAGCAGATAA CAATTGAAAT CTAATAAATC TGGCGGCGAG GGAGGGGGTG GATAGTG

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Figure 6. DNA sequence of the 1.5 kb enhancer region. Nucleotides are numbered relative to the transcriptional start site (+1). The sequence between -1080 and -1896 was previously reported by Nakajima *et al.* (15). The sequence between -1896 and -2547 is new. The 189 bp essential region is underlined. The 27 bp sequence identified in Figure 9 is shown in the box. The sequence data has been submitted to GenBank accession no. L33242.

demonstrate that the region between -1.08 and -2.6 can confer enhancer activity in a position and orientation independent manner and also functions with a heterologous promoter. In addition, transfection of p1.5FNCAT into HepG2 cells did not result in increased CAT activity relative to pFNCAT, while transfection into the human fibrosarcoma cell line, HT1080, did result in increased activity (data not shown). This shows that the effect is not species-specific and supports the cell-type-specificity of this enhancer region.

Localization of the active region within the 1.5 kb enhancer

The enhancer activity between -1.08 and -2.6 was further delineated by testing the activity of nested sets of fragments prepared by 5' and 3' exonuclease III deletions. Deletion from the 5' end generated four fragments terminating between -2.3 and -1.5 (Fig. 5A). Comparison of p1.9Δ5FNCAT with p1.5Δ5FNCAT shows that enhancer activity was completely lost with deletion of the segment between -1.9 and -1.5. The same region was identified by exonuclease treatment of the 3' end where enhancer activity was lost when the deletion was extended from -1.5 to -1.7 (p1.5Δ3FNCAT and p1.7Δ3FNCAT, Fig. 5B). In addition, removal of the 300 bp between -2.6 and -2.3 or the 220 bp between -1.08 and -1.3 resulted in an ~1.5-fold decrease in activity. However, the ends of the 1.5 kb fragment cannot act independently. Fragments including either the 5' end (p1.9Δ3FNCAT, Fig. 5B) or the 3' end (p1.5Δ5FNCAT, Fig. 5A), but lacking -1.5 to -1.9, do not show enhancer activity. Taken together, these data suggest that this is a complex region likely to contain multiple transcription factor binding sites. The fact that the CAT activity is completely abolished by deletion of sequences between -1.5 and -1.7 demonstrates that the minimal sequence for transcriptional activation is located in the center of the 1.5 kb

Figure 7. (A) Schematic showing the 189 bp fragment and the two sub-fragments; 65 bp and 124 bp. (B) Gel mobility shift assays using the three fragments in (A). Cell extracts used in the reactions are indicated above the lanes. ³²P-labelled probes used are shown above the brackets. Closed arrowheads indicate fibroblast-specific bands.

Figure 8. Gel shift competition assays to determine specificity of binding. The 189 bp fragment was ^{32}P -labelled and used as a probe. Reactions were carried out with nuclear extracts from 3T3 cells, HepG2 cells, Rat1 cells or rat liver. Competitor DNA (50-fold molar excess) used is shown above each lane. Closed arrowheads indicate competed bands in fibroblasts. Open arrows indicate competed bands in rat liver. ns, non-specific DNA.

fragment. Furthermore, a 346 bp fragment containing the sequence from -1.5 to -1.9 was active when placed upstream of the FN promoter (data not shown).

Figure 6 shows the complete sequence of the region between -1.08 and -2.6 . The region identified by deletion analysis is located between -1538 and -1726 (underlined).

Formation of fibroblast-specific protein-DNA complexes

The tissue-specific activity of the FN enhancer indicates that distinct proteins interact within this region in fibroblasts and liver. To test this hypothesis, gel mobility shift assays were used. Nuclear extracts were prepared from 3T3 and HepG2 cells as well as Rat1 fibroblasts and rat liver. The 189 bp enhancer fragment and two sub-fragments of 65 bp and 124 bp (Fig. 7A) were ^{32}P -labelled and used as probes in gel shift assays. Both the 189 and 65 bp probes exhibited binding to protein(s) in the 3T3 and Rat1 fibroblast extracts (Fig. 7B). Rat liver proteins also bound to the 189 bp and 65 bp probes, but formed distinct significantly smaller complexes from those seen with fibroblast extracts (Fig. 7B). The major band formed with the HepG2 extract migrated slower than the fibroblast band, but apparently does not represent a specific interaction (see Fig. 8). These data show that the 189 bp fragment and the 65 bp sub-fragment form a unique complex with protein(s) from fibroblasts.

To confirm the specificity of the protein-DNA complexes, competition assays with excess unlabelled fragments were performed. Binding to ^{32}P -labelled 189 bp fragment was competed with 50-fold molar excess of unlabelled 189, 65, 124 bp or non-specific DNA fragments as indicated in Figure 8. Protein binding from 3T3 and Rat1 extracts to the 189 bp probe was competed by the 189 bp fragment itself and the 65 bp sub-fragment, but was not affected by the 124 bp fragment or a non-specific competitor. Therefore, the fibroblast nuclear protein binding to the 189 bp fragment is specific and is due to sequences contained within the 65 bp sub-fragment. The 189 and 65 bp fragments also bind specifically to rat liver nuclear protein(s), but the protein pattern is distinct from that of fibroblasts (Fig. 8). The

HepG2 extracts do not appear to contain specific binding proteins as no bands are specifically reduced in the presence of competing fragments. The competition assays confirm the specificity of the fibroblast nuclear protein binding and the localization of the binding site to the 65 bp fragment.

Localization of the nuclear protein binding site

To further delineate the fibroblast nuclear protein binding site defined by the mobility shift assays, DNase I footprinting was carried out with a 346 bp end-labelled probe. This fragment (-1538 to -1883) was active in CAT assays (data not shown) and contains the 189 bp region used in the gel shifts described above. The probe was incubated with 3T3 cell nuclear extract followed by treatment with increasing concentrations of DNase I. Only one region was protected from digestion by nuclear protein binding (Fig. 9A) and spans a 27 bp sequence from -1662 to -1688 (Fig. 9B and Fig. 6) as determined by the parallel A>C and GA chemical sequencing reactions. This sequence lies within the 65 bp fragment which forms a distinct protein-DNA complex in fibroblasts. The 27 bp footprint sequence was compared to the transcription factor sites database (33) and no exact matches to RNA polymerase II factor consensus sequences were found.

Inactivation of the enhancer

In order to correlate enhancer activity with the presence of the protein binding site, two deletions were made within the 1.5 kb enhancer and tested by transfection of 3T3 fibroblasts. Deletion of a 165 bp fragment from -1737 to -1573 ($\text{p}\Delta 165\text{FNCAT}$), eliminating the 65 bp region identified in the gel shift assays, abolished the activity of the 1.5 kb enhancer region (Fig. 10). The active region was localized more specifically with a deletion from -1698 to -1666 ($\text{p}\Delta 33\text{FNCAT}$) which removes 23 nucleotides of the 27 bp protein binding site identified by footprinting. This small deletion also obliterated the activity of the 1.5 kb enhancer. The loss of activity upon deletion of the 33 bp sequence shows a direct correlation between this region and enhancer activity. Therefore, this novel binding site is responsible for the fibroblast-specific protein binding activity.

Figure 9. DNase I footprinting of a 346 bp fragment with 3T3 fibroblast nuclear extract. (A) Autoradiogram of the gel. A fragment from -1538 to -1883 was labelled with ^{32}P at the 5' end of the non-coding strand. This probe was incubated with no added protein (lane 1), 25 μg of 3T3 extract (lane 2) or 50 μg of 3T3 extract (lanes 3 and 4). Reactions were treated for 60 s with 0.8 U (lane 1), 1.5 U (lanes 2 and 3) or 3 U (lane 4) DNase I. A>C and GA ladders were made by chemical sequencing of the labelled probe. The protected region is noted by the bracket. (B) A schematic of the footprint relative to the entire 1.5 kb enhancer region. The 27 bp protected sequence is shown. The open box represents the 346 bp fragment used as the probe. The hatch marks represent the 65 bp fragment. The transcriptional start site is indicated by the arrow at +1.

DISCUSSION

We have identified a 27 bp fibroblast nuclear protein binding site at -1688 in the rat FN gene. This site is located within a 1.5 kb enhancer region which has activity in fibroblasts, but not liver cells. While fibroblasts and liver cells differed in the use of the region between -1.08 and -2.6, both cell types showed similar increases in activity with the p-3.9CAT plasmid compared to the p-3.2CAT plasmid. These data indicate different cell types use both overlapping and distinct elements to regulate FN expression. Although both cell types exhibited similar basal promoter levels, the overall pattern of CAT activity of the promoter fragments differed in 3T3 fibroblasts and HepG2 cells. While activity decreased in HepG2 cells with the addition of sequences upstream of the basal promoter, 3T3 cells showed a progressive elevation in promoter activity across the same region.

Exonuclease III deletion analysis of the 1.5 kb region demonstrated that it is complex and is likely to contain multiple regulatory sub-elements working together to give maximal activity. An essential region was identified between -1538 and -1726. In addition, small deletions at either end of the 1.5 kb sequence resulted in decreases in CAT activity, but the end

Figure 10. CAT activity of protein binding site deletion constructs. 1.5 kb enhancer plasmids with 165 bp (pΔ165FNCAT) or 33 bp (pΔ33FNCAT) deletions were transfected into 3T3 cells. CAT activity was normalized to pFNCAT (value = 1.0). Each column represents the mean value for an average of six experiments (minimum of three). The error bars represent standard deviations. Values were normalized for transfection efficiency by dot blot hybridization.

fragments alone were not sufficient to produce an increase in transcription. A complexity similar to the 1.5 kb enhancer has been demonstrated for several other enhancers. In a 1 kb lipopolysaccharide-responsive enhancer upstream of the human prointerleukin 1 β gene, a 200 bp region is needed for lipopolysaccharide-specific induction, but an additional 600 bp segment is necessary for maximal induction in monocytes (34). In 3T3 fibroblasts, a 1.1 kb enhancer in the first intron of the $\alpha 2(\text{I})$ collagen gene is active in 3T3 fibroblasts, but retains only partial activity when reduced to a 92 bp central fragment (35,36). Like the FN enhancer, the 5' end of the collagen enhancer is necessary for maximum activity, but is not active when tested alone (35).

Analyses of the transcriptional control of the FN gene have identified a number of promoter-proximal transcription factor consensus binding sequences (13,14). Several of these sites have been shown to interact with specific transcription factors involved in cAMP induction (16,37) and E1A-responsive repression of FN expression (15). Similar cAMP response element interactions have been observed with rat (14) and human (13) promoters which share 84% sequence identity. The mouse promoter (38) has not been tested, but shares 95% identity with the rat FN promoter (14). The 189 bp region identified here, which is significantly further upstream, also binds specific protein(s). This fragment and a 65 bp sub-fragment formed fibroblast-specific protein-DNA complexes in both mouse and rat fibroblast extracts suggesting conservation of the protein(s) and its binding site. These complexes were not formed with extracts of rat liver or a human hepatoma cell line confirming the cell-type-specificity.

Specific protein binding was also observed with rat liver extracts. However, the liver-specific pattern was distinct from fibroblasts. Possibly, the smaller liver complex might lack an essential component that is present in fibroblasts. Alternatively, the liver complex may contain a different set of proteins that either repress activity or enhance activity by interacting with liver-specific proteins binding outside the regions examined here.

DNase I footprinting using nuclear extracts from 3T3 fibroblasts demonstrated protection of a 27 bp region within the 65 bp fragment which showed specific fibroblast nuclear protein binding in gel shift assays. The nuclease hypersensitivity of the

sequence immediately 3' of the footprint is a hallmark of sequences adjacent to nuclear protein-binding regions. In addition, it is interesting to note that there is a pair of inverted repeats, GAATG and CATTC, beginning at the 5' end of the footprint and separated by 5 bp. These may provide potential sites for protein-DNA interactions. Comparison of the 27 bp sequence to the transcription factor sites database did not identify known elements. Therefore, we have identified a novel fibroblast nuclear protein binding site.

Elimination of 23 nucleotides of the nuclear protein binding site results in total loss of activity of the 1.5 kb enhancer region. Although binding sites are often active out of context when multimerized, multimers of a 27 or 65 bp sequence were not active alone (unpublished observations). These findings correlate the presence of the binding site with transcriptional activity and show that this region is essential but not sufficient for the enhancer activity. A similar case was described for the DR element within the human immunoglobulin kappa 3' enhancer which demonstrates protein binding in both footprinting and mobility shift assays. The element itself is not active (even as a tetramer), but mutations in the element decrease activity of the enhancer (39). In addition, LEF-1 protein binding sites do not activate transcription but facilitate interactions of separate binding sites by bending DNA (40,41).

The complexity of the 1.5 kb enhancer makes it likely that a combination of DNA binding factors, ubiquitous as well as cell type-specific, are involved in this regulation. Perhaps several factors binding within the 1.5 kb enhancer cooperate to stimulate FN expression in fibroblasts. The results presented here begin to provide insight into the complex regulatory mechanisms controlling FN expression and demonstrate the presence of a novel fibroblast nuclear protein binding site involved in tissue-specific regulation of the FN gene.

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