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DNA Binding Proteins From Keloid Fibroblasts Form Unique Complexes With the Human Fibronectin Promoter

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Keloids are pathological lesions characterized by enhanced expression of extracellular matrix molecules including fibronectin. A molecular dissection of the human fibronectin promoter was performed to identify DNA-protein interactions that correlate with altered fibronectin gene expression by keloid fibroblasts. DNA mobility shift patterns generated by nuclear extracts from skin, scar, and keloid fibroblasts were identical at a consensus CRE at −170 of the human fibronectin promoter whereas extracts from keloid fibroblasts formed complexes at a CRE/AP-1-like sequence at −415 that differed from those generated by skin and scar fibroblast extracts. The DNA-protein interactions identified at −415 were sensitive to altered serum concentrations in skin and scar but not keloid fibroblast cultures. The effects of forskolin and TGF-β on fibronectin expression correlated with changes in the DNA-protein complexes assembled on the −170 and −415 *cis* elements, respectively. Oligonucleotides containing consensus CRE and AP-1 sequences did not compete for binding of nuclear proteins to the CRE/AP-1-like domain at −415, suggesting that this is a unique *cis* element. These studies indicate that the human fibronectin promoter contains two *cis* elements on which related but nonidentical complexes form. Alterations in the complexes interacting with the sequence at −415 may be responsible for the differences in fibronectin gene expression among quiescent skin, mature scar, and keloid fibroblasts.

Wound healing Extracellular matrix Cyclic AMP response element

KELOIDS are tumor-like masses of connective tissue that develop in humans as a pathologic response to wounding [reviewed in (25,33)] and, like granulation tissue of normal wounds (21), exhibit elevated fibronectin content relative to both uninjured skin (19,40) and normal, mature scars (40). Dermal fibroblasts derived from skin, scar, and keloid maintain inherent differences in fibronectin biosynthesis and in steady-state content of fibronectin mRNA in a tissue culture environment (18,40). However, these differences are quantitative rather than qualitative markers, and scar fibroblasts are highly variable with respect to fibronectin expression (40), making differentiation of the scar and keloid fibroblast phenotype difficult. Thus, with the goal of identifying a qualitative marker for keloid fibroblasts, a molecular dissection of DNA-protein interactions on the fibronectin promoter in skin, scar, and keloid fibroblasts has been performed.

Although the *cis*-acting elements that mediate fibronectin expression by keloid fibroblasts are

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unknown, studies with other cell lines and strains have identified sequences in the human fibronectin promoter that control both basal and regulated expression (Fig. 1). Three cAMP-responsive elements (CREs or AP-1-like sequences) have been identified. The major CRE at −170 is a consensus CRE that also mediates basal transcription in HT1080 cells (10). The minor CREs at −260 and −415 are CRE half-sites (5). DNA fragments containing the minor CRE at −415 have been implicated in inhibition (4,26) as well as activation of fibronectin gene expression (5). An NF-1 like sequence is located between the major CRE and CCAAT box (12) but has not been identified as a functional *cis* element in any cells. The roles that these *cis* elements play in the regulation of fibronectin gene expression during normal wound healing and keloid formation are unknown and will be examined here.

A second goal of this study was to link signaling molecules involved in wound healing and/or regulation of fibronectin expression (TGF-β1, forskolin, components of serum) with downstream events (DNA–protein interactions on the fibronectin promoter) to identify signal transduction pathways that are altered in keloid fibroblasts and may be responsible for enhanced expression of the fibronectin gene.

The role of serum in wound healing is not well understood. However, many growth factors and cytokines present in serum are critical during all stages of the healing process (7). Furthermore, keloid fibroblasts grow to higher densities in low serum concentrations than do normal skin and scar fibroblasts (35), indicating an altered sensitivity to serum components. Whether this alteration affects pathways that regulate fibronectin gene expression is unknown and will be tested in this study.

TGF-β, which stimulates the synthesis of fibronectin and other extracellular matrix proteins by fibroblasts (16,31,32), is a potent modulator of tissue remodeling during wound healing (1,15,31,42), and inhibition of TGF-β action results in impairment of the healing process (20,22,39). Thus, like fibronectin expression, an imbalance in TGF-β expression may result in wounds that do not heal or wounds that produce excessive scars. It is possible that an alteration in a TGF-β signal transduction pathway is responsible for overexpression of fibronectin by keloid fibroblasts because keloid fibroblasts exhibit altered growth and metabolic properties in response to TGF-β (3,35). Like serum, TGF-β can mediate fibronectin expression at the level of transcription initiation (11,44), and its effects may be mediated by the consensus CRE at −170 (8). The effect of TGF-β1 on fibronectin gene expression and on DNA–protein interactions on the fibronectin promoter will be examined in this study to determine whether keloids exhibit altered responses to this regulator.

Unlike TGF-β, little is known about the role of cAMP during normal healing or whether a cAMP-mediated signal transduction pathway is altered in keloid fibroblasts. However, the human fibronec-

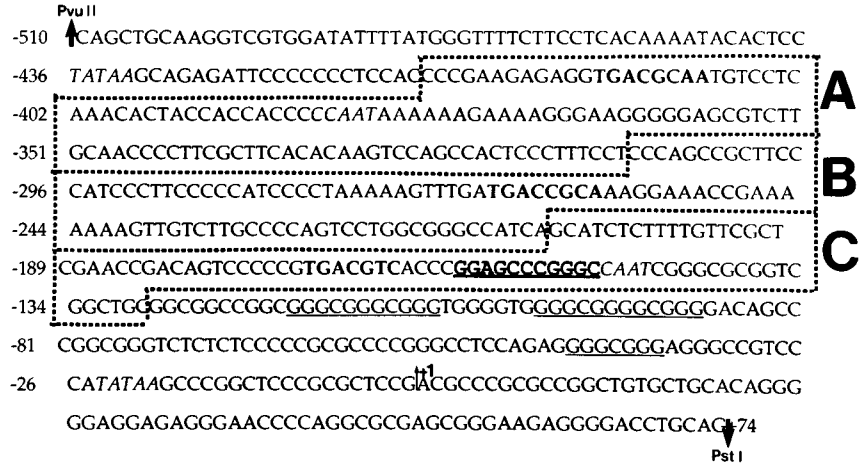


FIG. 1. The human fibronectin promoter. Human genomic fibronectin sequences from −506 to +74 were excised at PvuII and PstI sites from the vector 1.3 fn-CAT. Putative cis elements are shown. Italics: CCAAT and TATAA boxes; underline: SP-1/AP-2-like domains; bold: CRE/AP-1-like sequences; bold and underline: NF-1-like sequence. +1: transcription initiation site.

tin promoter contains three cAMP-responsive elements (CREs) (5), indicating that cAMP may function in basal and/or regulated fibronectin expression. Like serum, forskolin, a cAMP agonist, can activate (2,11) or inhibit (4,11,14) fibronectin biosynthesis, and the response may be cell type dependent. The positive and negative effects are both mediated in part by alterations at the level of fibronectin gene transcription (2,11,14). In this study, the effects of forskolin on steady-state fibronectin mRNA content and on DNA-protein interactions on the fibronectin promoter will be examined for skin, scar, and keloid fibroblast cultures.

The complexity of the human fibronectin promoter suggests regulatory input involving multiple signal transduction pathways that converge to modulate fibronectin synthesis at the level of transcription initiation. Thus, information concerning the interactions of transcription factors from skin, scar, and keloid with *cis*-acting elements in this promoter will serve to identify and distinguish gene regulatory pathways relevant to normal proliferation in skin or unique to keloid formation.

MATERIALS AND METHODS

Preparation of Nuclear Extracts From Fibroblast Cultures

Relevant information about the cell strains used in this study is listed in Table 1. Nuclear extracts were prepared essentially as described by Dignam et al. (13) and modified by Ritzenthaler et al. (30). Dermal fibroblasts were cultured in 10% fetal calf serum or in serum-free media containing 18 μM forskolin (Sigma, St. Louis, MO) in DMSO or 2 ng/ml recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) in 4 mM HCl, 0.1% bovine serum albumin (BSA) for 48 h. Control cultures were treated with vehicle alone. Nuclear extracts were prepared at 4°C or on ice. Cells from four or more 150-mm dishes were washed in PBS and pelleted three times at 350 × *g* for 5 min, resuspended in 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml chymostatin, 10 μg/ml pepstatin A, 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, incubated 10 min, and pelleted at 700 × *g* for 10 min. Cells were then resuspended in the above buffer, lysed in a Dounce homogenizer (tight pestle) for 15 strokes, and microfuged at full speed for 20 min. The nuclei were then resuspended in 200 μl 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml chymostatin, 10 μg/ml pepstatin A, 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, stirred for 30 min, microfuged as above, and the supernatant was dialyzed 4 h to overnight (Spectrum Medical Industries, Los Angeles, CA, 10,000 MW cutoff) in 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF, 3 μg/ml chymostatin, 3 μg/ml pepstatin A, 0.1 μg/ml leupeptin, 0.2 μg/ml antipain, 1 μg/ml benzamidine. Dialyzed supernatant was microfuged, frozen in liquid nitrogen, and stored at −80°C. Protein concentration was determined by the BioRad (Hercules, CA) protein assay.

Preparation of Probes and Competitor DNAs for DNA Mobility Shift Assays

DNA probes were synthesized by PCR using the human fibronectin promoter plasmid 0.6 fn-CAT as template (Fig. 1) and the following primer pairs: (A) 5′ primer: 5′-CCCGAAGAGAGGT GACGCAA-3′ and 3′ primer: 5′-AGGAAAGG GAGTGGCTGGAC; (B) 5′ primer: 5′-CCCA GCCGCTTCCCATCCCT-3′ and 3′ primer: 5′-TGATGGCCCGCCAGGACTGG-3′ and (C) 5′ primer: 5′-GCATCTCTTTTGTTCGCTGC-3′ and 3′ primer: 5′-GCAGCCGACCGCGGCC GATT-3′. PCR conditions were one cycle at 94°C for 1 min, 35 cycles at 94°C for 15 s, 55°C for 15 s, 72°C for 30 s, and one cycle at 72°C for 5 min. PCR products were ethanol precipitated, purified on NA45 nitrocellulose (Schleicher and Schuell, Keene, NH), and end-labeled with [γ-^{32}P]ATP (> 5000 Ci/mmol, Amersham) using polynucleotide kinase (Boehringer Mannheim or Gibco). All probes were separated from unincorporated nucleotides on G-50 Sephadex spin columns, phenol/chloroform extracted, and ethanol precipitated. Incorporation was determined by TCA precipitability.

DNA Mobility Shift Assays

DNA mobility shift assays (DMSA) were performed as described by Singh et al. (41) and modified by Rider and Peterson (29). Nuclear extract (5 μg) was incubated with 25,000 cpm labeled probe and 8 μg unlabeled poly(dI-dC) (Pharmacia, Uppsala, Sweden) as nonspecific competitor in DMSA binding buffer (100 mM KCl, 15 mM Tris-HCl, pH 7.5, 5 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, 12% glycerol) to a final volume of 30 μl. Binding reactions were incubated at 30°C

TABLE 1
CELL STAINS USED IN THIS STUDY AND PATIENT INFORMATION

Age	Sex	Race	Tissue Collected	Sample Used for: DMS	NOR
35	F	Black	keloid	X	X
49	F	Black	keloid and normal skin	K	K
24	M	Hispanic	keloid	X	X
20	F	Black	keloid and normal skin	X	
22	F	Black	keloid	X	X
26	F	Black	keloid	X	X
18	F	Black	keloid	X	X
53	M	Black	normal scar	X	X
51	M	Caucasian	normal scar		X
3	M	Caucasian	normal scar		X
11	F	Caucasian	normal scar and skin	X	X
21	F	Caucasian	normal scar	X	X
35	F	Black	normal scar and skin	sk	sk
24	F	Hispanic	normal scar and skin	X	
28	F	Caucasian	normal skin		X
18	F	Caucasian	normal skin	X	X
41	F	Black	normal skin	X	X

K: only keloid tissue was available for this experiment. sk: only normal skin was available for this experiment. DMS: DNA mobility shift assay. NOR: Northern analysis.

for 20 min and resolved on 4% nondenaturing polyacrylamide gels (1.5 mm thick). Gels were dried and exposed to Kodak XAR5 film at −80°C.

Specific unlabeled competitor DNAs were added to some reactions at 100-fold molar excess or greater. These DNAs included: the sequences that were used as probes, annealed oligonucleotides including the putative NF-1 site in the human fibronectin promoter (5′-GATTGC**GGAGCCC GGGCCAA**TGAGAGC-3′), a CCAAT-containing sequence (5′-GATTGC**CCAAT**GAGAGC-3′), the A-CRE in the human fibronectin promoter (5′-**GAGAGGTGACGCAATGTCCTCA AACACTAC**-3′), and the following oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, CA): AP-1 (5′-CGCTTGATGACTCAGCCGG AA-3′), consensus CRE (5′-AGAGATTGC CTGACGTCAGAGAGCTAG-3′), and mutant CRE (5′-AGAGATTGCCTGTGGTCAGAGAG CTAG-3′). (Sequences derived from the human fibronectin promoter are in bold. Consensus *cis* element sequences are underlined.)

Northern Analysis

Fibroblasts were cultured continuously in 10% FCS then switched to 0.5%, 2%, or 10% FCS or serum-free media containing 18 μM forskolin (Sigma) in DMSO or 2 ng/ml recombinant human TGF-β1 (R&D Systems) in 4 mM HCl, 0.1% BSA for 48 h. Control cultures were treated with vehicle alone. Total RNA was isolated by the method of Chirgwin et al. (6) and quantitated by spectrophotometry. Total RNA (2 μg) was resuspended in 10 μl Northern loading buffer (50% formamide, 10% glycerol, 2 M formaldehyde, 0.08% bromphenol blue, 0.2 mM EDTA), denatured at 85°C for 10 min, and resolved on 1% agarose gels containing 20 mM MOPS, 2 M formaldehyde, 5 mM NaOAc, 1 mM EDTA, pH 7. The gels were washed in distilled H_2O, denatured with 50 mM NaOH, 10 mM NaCl, neutralized with 0.1 M Tris, pH 8, and then RNA was transferred onto Biotrans™ nylon membrane (ICN) using the VacuGene vacuum transfer apparatus (Pharmacia). RNA was cross-linked to the blot with a Fisher UV Crosslinker. The blots were prehybridized for 2 h or more at 42°C in hybridization solution [50% formamide, 5 × SSPE (20 × SSPE = 3.6 M NaCl, 0.2 M NaH_2PO_4 × H_2O, 0.02 M EDTA, pH 7.4], 5 × Denhardt's, 0.1% SDS, 200 μg/ml denatured salmon sperm DNA. Nick-translated probes were synthesized using 100 ng pHF154, a plasmid containing 2 kb of human fibronectin cDNA sequence (kindly provided by Dr. Alberto Kornblihtt, INGEBI-CONICET, Buenos Aires, Argentina), and pB, a plasmid containing human

18S rRNA sequence (kindly provided by Dr. J. Sylvester, University of Pennsylvania). Probes were synthesized using the Nick Translation Kit (Amersham) and [α-^{32}P]dCTP (Amersham or ICN). The blots were then hybridized with the fibronectin probe overnight at 42°C in hybridization solution containing 1×10^6 cpm/ml denatured probe. The next day, blots were washed 4 × 15 min at 50°C in 2 × SSC, 0.1% SDS. Autoradiography was performed as described above. Blots were stripped by washing three times in boiling 0.1% SDS and then reprobed for 18S.

RESULTS

Nuclear Extracts From Skin, Scar, and Keloid Fibroblasts Interact Differently With the Human Fibronectin Promoter

The interactions of nuclear factors derived from skin, scar, and keloid fibroblasts with the fibronectin promoter were examined using a PvuII/PstI fragment containing human fibronectin promoter sequence from −510 to +69 (Fig. 1). This was derived from the plasmid, 1.3 fn-CAT, a gift of Dr. Douglas Dean (Washington University, MO). In addition to a TATAA box at −25 and a CCAAT box at −150, this segment contains another consensus CCAAT box −385 and a TATAA box at −436. It was not known for skin, scar, and keloid fibroblasts whether fibronectin gene transcription could be initiated at alternative sites guided by the other CCAAT and TATAA boxes. However, S1 nuclease analysis performed with RNA derived from skin, scar, and keloid fibroblasts indicated that these cells all initiate fibronectin gene transcription at the same site (data not shown). Thus, the PvuII/PstI fragment of the fibronectin gene contains 500 bases of promoter sequences relevant as a probe of fibronectin gene regulation for all three cell types.

Previous studies have indicated that synthesis of fibronectin by keloid fibroblasts is due to altered gene transcription (27). However, transfection of primary cultures of human dermal fibroblasts with reporter gene constructions is inefficient, precluding quantitative analysis of fibronectin gene expression in keloids (J. Sible, unpublished data). To identify *cis*-acting elements in the fibronectin promoter that could mediate this altered expression, DNA mobility shift assays were performed. Nuclear extracts were prepared from skin, scar, and keloid fibroblasts cultures grown continuously in 10% fetal calf serum (FCS), and these extracts were allowed to interact with ^{32}P-labeled PCR fragments derived from the human fibronectin promoter (Fig. 1) in DNA mobility shift assays. Fragment A contains a CRE/AP-1-like sequence that has been identified as a minor CRE in some cell lines and a consensus CCAAT box (5). Fragment B contains another minor CRE (5). Fragment C contains the major CRE, an NF-1-like sequence, and the CCAAT box (10).

The DNA mobility shift assays indicate that the complexes formed by nuclear extracts and the A fragment differ among skin, scar, and keloid fibroblasts (Fig. 2A). Three shifted bands are generated by the keloid extract whereas the skin and scar extracts favor the upper two of these bands as well as a fourth band that migrates between the first and the second. In all studies, extracts from keloids generated these three shifted bands whereas extracts from skin and scar favored one or more of the upper bands. The appearance of the fourth band in skin and scar varied from experiment to experiment (compare Figs. 2A and 3A). None of the nuclear extracts generated complexes with the B fragment (Fig. 2B), whereas all of the extracts generated similar complexes with the C fragment (Fig. 2C). Thus, DNA-protein interactions within the A fragment may mediate differences in fibronectin gene expression between skin, scar, and keloid. The abundant complex that is formed by all nuclear extracts with the C fragment suggests that this domain binds regulators of transcription, but not necessarily regulators that differentiate expression of fibronectin among these fibroblasts. This fragment served as a positive control for the quality of nuclear extracts and binding reactions in further studies.

In Keloid Fibroblasts, Nuclear Complexes on the A Fragments of the Fibronectin Promoter Are Insensitive to Varied Concentrations of Serum

To determine whether serum, a regulator of fibronectin expression, altered the DNA-protein interactions identified in Fig. 2, DNA mobility shift assays were performed with nuclear extracts from fibroblasts cultured in 2% or 10% FCS (Fig. 3). Both the A fragment of the human fibronectin promoter (Fig. 3A), which previously demonstrated differential DNA-protein interactions, and the C fragment (Fig. 3B), which did not, were used as probes. [Studies were also performed with the B fragment as probe, but no shifted bands were detected with nuclear extracts from cultures

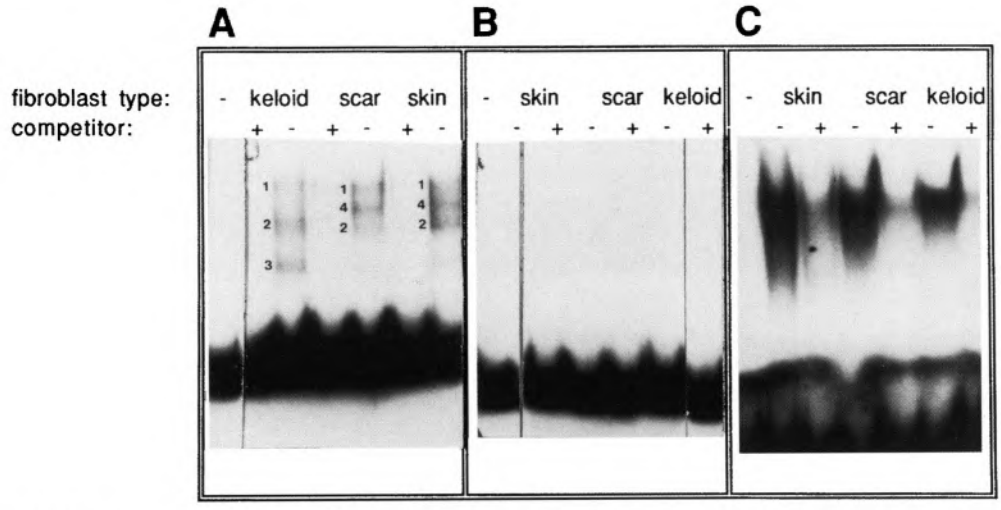


FIG. 2. DNA mobility shift assays of nuclear extracts from skin, scar, and keloid fibroblasts binding fragments of the human fibronectin promoter. Interactions of nuclear extracts with ^{32}P-labeled A, B, and C fragments are shown. The discrete bands generated by incubation of the A probe with nuclear extracts are numbered 1–4. +: assays performed in the presence of 100-fold molar excess unlabeled probe as competitor.

grown in 2% or 10% FCS (data not shown).] When skin, scar, and keloid fibroblasts were cultured in 2% FCS, the gel shift patterns with the A fragment were identical for all three cell types (Fig. 3A). Three bands were apparent, with the middle band of greatest intensity. However, when cultured in 10% FCS, only keloid fibroblasts demonstrated the same pattern as the 2% FCS cultures whereas the skin and scar samples favored the upper two bands and sometimes a fourth band as well. This is consistent with data shown in Fig. 2 in which the gel shift pattern for keloid samples differed from that of skin and scar samples when fibroblasts were cultured continuously in 10% FCS. These studies indicate that skin and scar cultures respond to different serum concentrations by altering DNA–protein interactions at the CRE/AP-1-like *cis* element within the A fragment whereas keloid fibroblasts are insensitive to this effect of serum.

DNA mobility shift assays performed with the C fragment as probe demonstrated that the band shift pattern at this site is neither cell type nor serum dependent (Fig. 3B). This is consistent with the hypothesis that the consensus CRE in this fragment mediates basal expression of fibronectin by all of these fibroblasts. These data also indicates that the quality of nuclear extracts prepared from fibroblasts grown at different serum concentrations is not altered by some general metabolic effect.

Keloid Fibroblasts Exhibit a Biphasic Response to Serum With Respect to Steady-State Content of Fibronectin mRNA

To correlate the differential DNA/protein interactions identified with changes in fibronectin gene expression in response to serum, skin, scar, and keloid fibroblasts were cultured in 10% FCS continuously and then shifted to 0.5%, 2%, or 10% FCS for 48 h. Total RNA was then prepared, and steady-state content of fibronectin mRNA was determined by Northern analysis. These studies revealed considerable differences among the fibroblast strains, probably reflecting heterogeneity within the human population. However, several trends were apparent. In general, fibronectin expression was stimulated in normal skin fibroblasts by 2% relative to 0.5% serum with no further increase in response to 10% FCS (Fig. 4A, B). For most strains of normal scar fibroblasts, steady-state content of fibronectin mRNA and concentration of serum demonstrated a direct correlation (Fig. 4A, B). The strains of keloid fibroblasts demonstrated the most striking response to serum concentration. Fibronectin expression was inhibited by 2% relative to 0.5% FCS whereas expression in 10% FCS was similar that in 0.5% FCS (Fig. 4A, B). These data suggest that serum contains substances that can both stimulate and inhibit fibronectin expression, that these substances have different critical concentrations, and that ke-

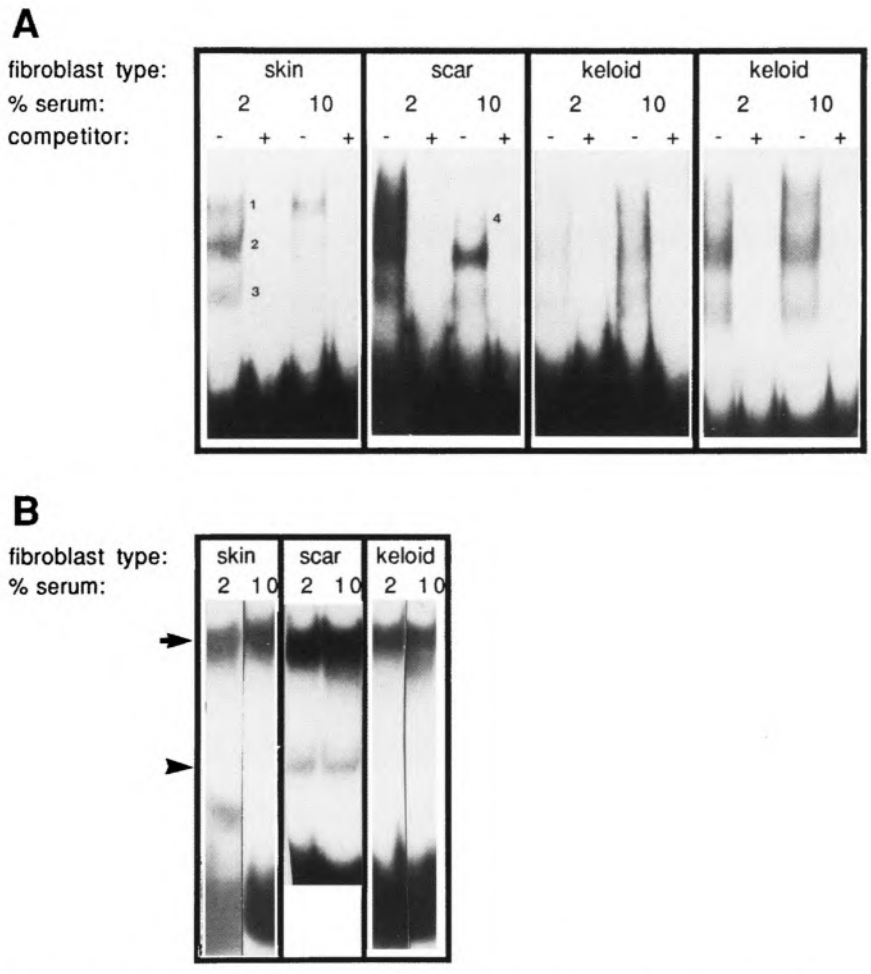


FIG. 3. DNA mobility shift assay of fibronectin promoter fragments interacting with nuclear extracts from skin, scar, and keloid fibroblasts cultured in 2% or 10% FCS. (A) Assays performed with the A fragment as probe. The retarded bands are labeled 1–4. +: assays performed in the presence of 100-fold molar excess unlabeled probe as competitor. (B) Assays performed with the C fragment as probe. Arrow: retarded band identified in all assays. Arrowhead: second band that is present or absent in all samples in an assay-dependent manner.

loid, skin, and scar fibroblasts exhibit differential sensitivities to these substances. However, because of the complexity in the response of each of the fibroblast types to serum, no general correlations can be made regarding the effect of serum on steady state levels of fibronectin RNA and on DNA–protein interactions within the fibronectin promoter.

Activation of Fibronectin Expression by TGF-β1 and Inhibition by Forskolin Are Associated With Altered Complexes Assembled on the C and A Fragments of the Fibronectin Promoter, Respectively

The effects of TGF-β1 and forskolin, two other known regulators of fibronectin expression, on DNA–protein interactions on the fibronectin promoter were examined by DNA mobility shift assays with the A and C fragments. Because serum has previously been shown to block or diminish the effects of TGF-β1 on fibronectin expression (3,11,28), fibroblasts were cultured continuously in 10% FCS and then switched to serum-free media with vehicle alone, 2 ng/ml TGF-β1, or 18 μM forskolin, a cAMP agonist, for 48 h, and then nuclear extracts were prepared. In DNA mobility shift assays with the A fragment, no qualitative differences were apparent. In the absence of serum (Fig. 5A, −), three major bands were present in a pattern similar to that seen in all fibroblasts cultured in 2% FCS and keloid fibroblasts cultured in 10% FCS (Fig. 5A, S). Although this

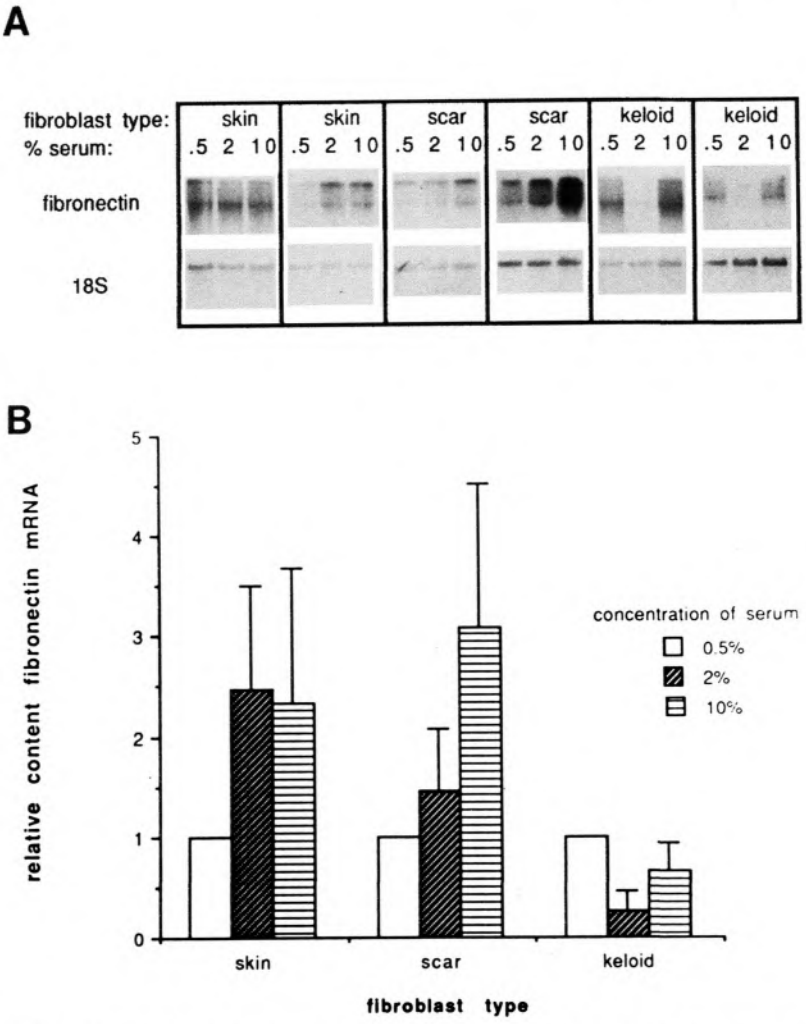


FIG. 4. Northern analysis of steady-state content of fibronectin mRNA in response to serum. Fibroblasts from skin, scar, and keloid were cultured in 0.5%, 2%, or 10% FCS. (A) Representative Northern blots were probed for fibronectin and then stripped and reprobed for 18S rRNA. The fibroblasts used in these studies were derived from six different patients. Because different autoradiographic exposures are shown for each fibroblast strain, relative intensities should be compared within a sample but not between different samples. (B) Densitometric analysis of Northern blots. The optical densities of fibronectin bands normalized to those of 18S are graphed for three skin, three scar, and three keloid samples. The relative content of fibronectin mRNA is set to 1 for 0.5% FCS samples. Error bars: SEM.

pattern of shifted bands was unchanged by treatment of cultures with forskolin (Fig. 5A, F), a striking quantitative increase in the complexes assembled on the A CRE by skin, scar, and keloid fibroblast nuclear extracts was observed. A similar increase was not observed in complexes assembled on the C CRE (Fig. 5B), indicating that this not a general enrichment in transcription factors, but rather a specific increase in factors that interact with the A CRE or possibly an increased affinity of these factors for the A CRE. Treatment with TGF-β1 produced neither a qualitative nor quantitative effect on the complexes that assembled on the A CRE.

DNA mobility shift assays with the C fragment indicated that forskolin had no effect on the content or mobility of complexes interacting with the C CRE (Fig. 5B, F). However, TGF-β1 treatment resulted in the appearance of two faster migrating bands (Fig. 5B, T, arrows). Like the effect of forskolin on interactions with the A fragment, this result was similar for skin, scar, and keloid fibroblasts. The appearance of these two additional bands seems to be a qualitative rather than a

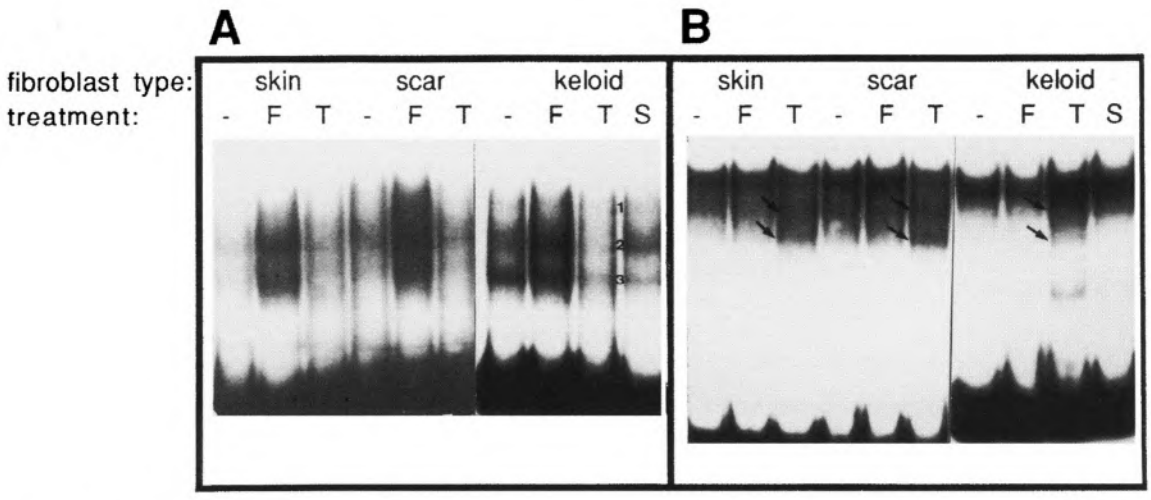


FIG. 5. DNA mobility shift assay of fibronectin promoter fragments interacting with nuclear extracts from skin, scar, and keloid fibroblasts cultured in the presence of forskolin or TGF-β1. Cells were treated for 48 h in serum-free media with no treatment (−), 18 μM forskolin (F), 2 ng/ml TGF-β1 (T), or continuously in 10% fetal calf serum (S). (A) Assays performed with the A fragment as probe. The retarded bands are labeled 1–3. (B) Assays performed with the C fragment as probe. Arrows: additional bands induced by TGF-β1 treatment.

quantitative effect because the intensity of the upper, previously identified band was not increased.

TGF-β1 Is an Activator and Forskolin an Inhibitor of Fibronectin Gene Expression in all Dermal Fibroblasts

Northern analysis was then performed to determine whether changes in DNA–protein interactions on the fibronectin promoter in response to forskolin and TGF-β1 correlated with altered steady-state levels of fibronectin mRNA. Fibroblasts were cultured continuously in 10% FCS and then switched to serum-free media with vehicle alone, 2 ng/ml recombinant human TGF-β1, or 18 μM forskolin for 48 h. Representative Northern blots are shown in Fig. 6. TGF-β1 stimulated fibronectin expression by skin, scar, and keloid fibroblasts. The stimulatory effect in keloid fibroblast cultures was less than that seen in skin and scar fibroblasts (Fig. 6). This may reflect the fact that basal rates of fibronectin expression are maximal in keloids and cannot be stimulated further. In contrast to TGF-β1, forskolin inhibited fibronectin expression by skin, scar, and keloid fibroblasts (Fig. 6). This inhibition was apparent even at lower concentrations of forskolin (7 vs. 18 μM for scar) and occurred within 24 h (24 vs. 48 for keloid).

These data identify TGF-β1 and forskolin as positive and negative regulators, respectively, of fibronectin expression by all dermal fibroblasts, and suggest that common signal transduction pathways are engaged in each fibroblast type because similar interactions with the fibronectin promoter are observed. These data support a hypothesis that forskolin mediates negative regulation of fibronectin expression via the A CRE and that TGF-β1 mediates positive regulation via the C CRE or another *cis* element within the C fragment.

Interaction of Nuclear Factors With a CRE/AP-1-Like Sequence Is Responsible for the Differential Gel Shift Patterns Generated by Extracts From Skin, Scar, and Keloid Fibroblasts

To more precisely map the *cis*-acting elements that bound to nuclear factors in the A and C fragments of the fibronectin promoter, DNA mobility shift assays were performed using unlabeled oligonucleotides as competitor DNAs (Fig. 7). A nuclear extract prepared from scar fibroblasts grown in 2% FCS was incubated with the ^{32}P-labeled A fragment in the presence or absence of competitor DNAs at greater than 100-fold molar excess. This extract was selected because the three major bands previously identified in skin, scar, and keloid are present in a distinct pattern (Fig. 3A). The shifted bands are completely competed by the entire A fragment ('A'), confirming the specificity of these

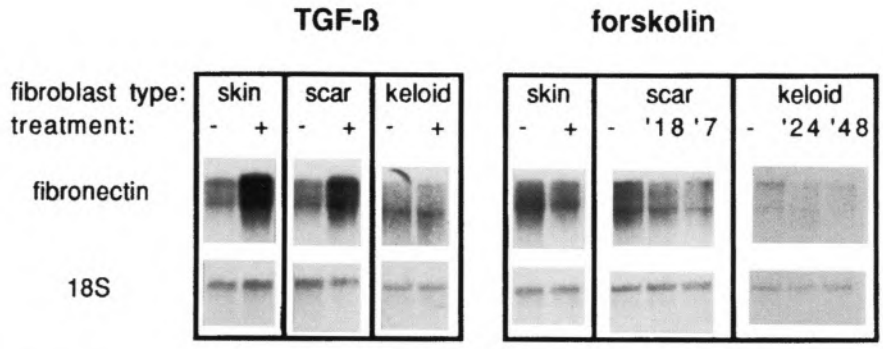


FIG. 6. Northern analysis of steady-state fibronectin mRNA content in fibroblasts treated with TGF-β1 and forskolin. Fibroblasts were cultured for 48 h in serum-free media alone (−) or with 2 ng/ml TGF-β1 or 18 μM forskolin. An additional scar culture was treated with 7 μM forskolin, and an additional keloid culture was treated with forskolin for 24 rather than 48 h. Blots were probed for fibronectin mRNA and then stripped and reprobed for 18S rRNA. Representative blots from several different experiments are shown. Relative intensity of autoradiographs should be compared within a particular sample but not between different samples.

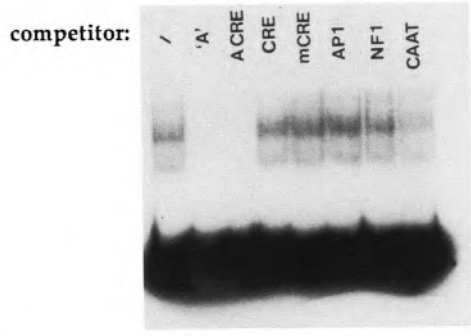
shifts. A 30 base pair oligonucleotide containing the CRE/AP-1-like sequence within the A region also completely competes for all of the shifted bands (A CRE, Fig. 7A, B). Oligonucleotides containing a consensus CRE, a mutant CRE, a consensus AP-1, an NF-1-like sequence, and a consensus CCAAT box do not compete for binding of the A fragment to nuclear factors (Fig. 7A, B). These data indicate that the A region contains a unique *cis* element, the A CRE, previously identified functionally as a minor CRE (5). The fibronectin A CRE forms complexes with nuclear factors that are distinct from complexes formed at consensus CRE and AP-1 *cis* elements. The CCAAT box in the A fragment appears not to be required for binding of these nuclear complexes, although an interaction between CCAAT binding factors and factors binding to the A CRE cannot be precluded.

Similar DNA mobility shift assays were performed with the C fragment as probe and unlabeled DNAs as competitor (Fig. 7C). An oligonucleotide containing a consensus CRE competed with the C fragment for the shifted band. No competition was observed by oligonucleotides containing a mutant CRE, the NF-1-like sequence in the fibronectin promoter, and a CCAAT box. Even at more than 500-fold excess (5 × , Fig. 7C), the NF-1- and CCAAT-containing oligonucleotides did not compete with the C fragment for binding to nuclear extracts. Unlabeled A fragment partially competed with the C fragment for binding to factors in the nuclear extract. These data confirm previous studies that identified a CRE within this region (10) and indicate that this CRE and not the NF-1-like or CCAAT sequences is responsible for the DNA–protein complex identified in the DNA mobility shift assays.

DISCUSSION

Excessive deposition of connective tissue associated with overexpression of extracellular matrix genes is both a clinical marker for keloids and a physiologic marker for the phenotype of cultured keloid fibroblasts. In these studies, fibronectin gene expression was used as a probe to dissect signal transduction pathways that are altered in keloids and may lead to the enhanced expression of several extracellular matrix genes. The results of previous studies with cultured fibroblasts suggest that a defect in one or more signal transduction pathways is responsible for the overexpression of extracellular matrix genes by keloids (3,34–38,45). Other studies demonstrate that accumulation of fibronectin in keloids is due to enhanced gene transcription (27). The results presented here link these previous observations by demonstrating that nuclear factors from skin, scar, and keloid fibroblasts interact differently with a unique *cis*-acting element in the human fibronectin promoter. This is the first evidence that altered gene expression in keloids is associated with a specific DNA–protein interaction. The effects of three known regulators of fibronectin expression: serum, TGF-β1, and forskolin, on steady-state content of fibronectin mRNA and on the transcription factor complexes assembled on the fibronectin promoter were examined here. The

A



B

A CRE	GAGAGG*TGACGCAA*TGTCCTCAAACACTAC
CRE	AGAGATTGCC*TGACGTCA*GAGAGCTAG
mCRE	AGAGATTGCC*TGTGGTCA*GAGAGCTAG
AP-1	CGCTTGA*TGACTCA*GCCGGAA

C

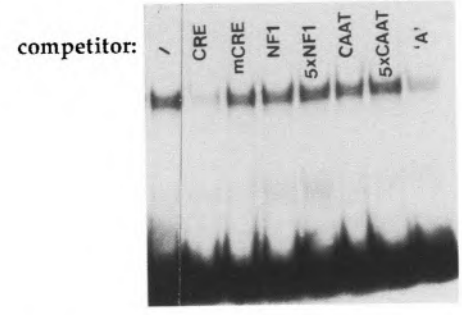


FIG. 7. DNA mobility shift assays in the presence of unlabeled competitor DNAs. (A) Nuclear extracts from scar fibroblasts grown in 2% fetal calf serum were incubated with ^{32}P-labeled A fragment in the absence (/) or presence of unlabeled competitor DNAs. Competitors included: the entire A fragment ('A') and oligonucleotides containing the A CRE, a consensus CRE, a mutant CRE (mCRE), a consensus AP-1, an NF-1 like sequence, and a CCAAT box. (B) The A CRE and other oligonucleotides used as competitor. The putative *cis*-acting elements are italicized. Sequences in the consensus CRE and AP-1 that are identical to those in the A CRE are underlined. (C) Nuclear extracts from keloid fibroblasts grown in 2% fetal calf serum were incubated with ^{32}P-labeled C fragment in the absence (/) or presence of unlabeled competitor DNAs at 100-fold molar excess or greater. 5 × : samples contained greater than 500-fold excess competitor DNAs.

results indicate that keloid fibroblasts possess a biphasic, altered response to increased concentrations of serum and that all dermal fibroblasts are sensitive to stimulation of fibronectin expression by TGF-β1 and inhibition of fibronectin expression by forskolin.

In these assays, two *cis*-acting elements within the proximal human fibronectin promoter interacted with nuclear factors. Similar complexes formed with nuclear factors from skin, scar, and keloid fibroblasts on a consensus CRE (the C CRE) located at −170. The abundance of complexes assembled on this *cis* element in skin, scar, and keloid fibroblasts may indicate that interactions with this CRE are responsible for the high levels of fibronectin synthesized by all dermal fibroblasts in culture. This is consistent with the role of the major CRE in mediating serum stimu-

lation as well as basal fibronectin expression by HT1080 cells (9). Differential interactions of nuclear extracts from skin, scar, and keloid cultures were observed with the CRE/AP-1-like sequence at −415 (the A CRE). This *cis* element has previously been demonstrated to mediate stimulation of fibronectin gene expression by forskolin in HT1080 cells (5). However, repression of fibronectin expression by forskolin in a bovine granulosa cell line (4) has also been mapped to DNA fragments containing this *cis* element. DNA footprinting studies have also demonstrated that nuclear factors bind this site in adenovirus E1a-transformed fibroblasts in which fibronectin expression is repressed (26). Thus, it is likely that the A CRE can act as both a stimulator and repressor of fibronectin transcription depending on the cell type and the regulatory pathway engaged.

Steady-state content of fibronectin mRNA in keloid fibroblasts was reduced dramatically by 2% FCS and restored to basal levels or greater by 10% FCS. These data suggest that serum contains both activators and inhibitors of fibronectin expression that possess distinct critical concentrations. Skin, scar, and keloid fibroblasts possess different sensitivities to these regulators. These data are supported by the DNA mobility shift studies with the A fragment of the fibronectin promoter, which demonstrate that DNA–protein interactions at this site are altered at higher serum concentrations in skin and scar whereas keloids are refractile to this effect. One hypothesis to explain these data is that the A CRE mediates negative feedback regulation at higher serum concentrations (10%) in skin and that keloids are insensitive to this effect. The presence of three retarded bands in assays with 2% FCS samples suggests that several sets of transcription factors are capable of assembling on the A CRE. The complexes favored in the 10% FCS samples may be those that downregulate fibronectin expression. Activators of fibronectin gene expression present in serum may act via a different site such as the C CRE, which has already been shown to mediate this effect for HT1080 (9). The net effect of activators that function at one site (the C CRE) at a low critical concentration (2% FCS) and inhibitors that function at a different site (the A CRE) at a higher critical concentration (10% FCS) would be activation of fibronectin expression in 2% FCS and feedback regulation to prevent further activation in 10% FCS as is seen for normal skin fibroblasts. These regulatory pathways may be intact in normal skin fibroblasts and altered in scar and keloid fibroblasts. The inhibition of fibronectin expression by keloid fibroblasts in 2% FCS does not fit simply into this model and suggests that keloids possess altered sensitivity to yet another component of serum that impinges upon fibronectin gene expression. Identification of this factor may suggest a new therapy for controlling the exuberant deposition of extracellular matrix by keloids, one that takes advantage of the unique keloid phenotype.

Unlike serum, TGF-β1 increased the steady-state content of fibronectin mRNA in normal skin, mature scar, and some keloid fibroblasts. As has previously been demonstrated, keloid fibroblasts were less sensitive to this effect (3), suggesting that the signal transduction pathway mediated by keloids may already be partially engaged. Alternatively, keloids may synthesize fibronectin at near maximal rates in the absence of TGF-β1 and cannot be stimulated further. The increase in steady-state levels of fibronectin mRNA in response to TGF-β1 was associated with the appearance of two additional retarded bands in DNA mobility shift assays assembled on the C fragment of the fibronectin promoter. These bands could be due to an interaction with the CRE, NF-1-like sequence, CCAAT box, or a previously unidentified *cis* element. We favor the hypothesis that these bands represent alternative complexes with the C CRE because binding of proteins to another site should cause supershifted bands that include both the CRE complex and the additional complex. However, it is possible that binding of factors to a CRE and to another *cis* element within the C fragment are mutually exclusive. TGF-β1 can mediate its effects on gene transcription via NF-1-like sequences (30), but for the human fibronectin promoter, TGF-β1 has been reported to act via the C CRE (8).

Treatment with forskolin decreased steady-state levels of fibronectin mRNA in skin, mature scar, and keloid fibroblasts. This effect was apparent even at lower concentrations than are typically used (7 μM; Fig. 2). Most previous studies in which forskolin was demonstrated to activate fibronectin gene expression were performed with immortal cell lines (4,11), and because these cells are not phenotypically normal, particularly with respect to growth and synthesis of extracellular matrix, the physiologic relevance of the data is questionable. For example, HT1080 fibrosarcoma cells, which synthesize very low basal levels of fibronectin, contain an activated *ras* gene that may affect signal transduction via cAMP (10). Thus, the most physiologically relevant data may be that obtained with normal human fibroblasts (11), cytotrophoblasts (14), and liver (24,43). Taken together, these data suggest that the major cAMP response element (C CRE) mediates basal tran-

scription but that exogenous cAMP inhibits transcription, possibly through the A CRE.

The data presented here suggest the following model for transcriptional regulation of fibronectin expression by the CRE-like elements in the human fibronectin promoter (Fig. 8). The consensus CRE (C CRE) may function as a regulator of basal fibronectin expression as has previously been shown for this gene (10,23) and for CREs in other genes (17). This is consistent with the role of the C CRE in promoting the binding of transcription factors to the adjacent CCAAT box (24,43). The C CRE may also mediate positive regulation of fibronectin expression by serum and TGF-β1 (8,9). Negative regulation of fibronectin by forskolin and by higher serum concentrations may be mediated by the A CRE. Although keloid fibroblasts are sensitive to the effects of TGF-β and forskolin, they may be insensitive to the feedback inhibition of higher serum concentrations. This is consistent with their altered pattern of DNA–protein interactions at the A CRE. Similarly, human fibrosarcoma cells, which increase transcription of the fibronectin gene in response to forskolin (11), may possess defects in cAMP-mediated signal transduction pathways that act on these CREs, resulting in activation rather than inhibition of fibronectin gene expression.

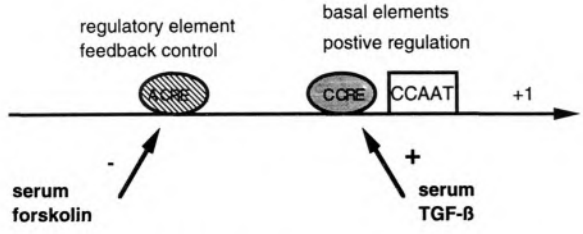


FIG. 8. A model for regulation of fibronectin gene expression by the A and C CREs. The C CRE, which regulates interactions with the CCAAT box, mediates basal expression as well as positive regulation by serum and TGF-β1. The A CRE serves as a feedback control switch and mediates negative regulation by higher concentrations of serum and by forskolin. Keloid fibroblasts are insensitive to the effects of higher serum concentrations on the A CRE, which may account in part for their enhanced expression of the fibronectin gene.

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