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Modulation of Transcription of the Rat Fibronectin Gene by Cell Density

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Abstract The fibronectin (FN) gene is under complex regulatory control in vitro and in vivo. Sequences from the rat FN gene directed efficient expression of a *lacZ* reporter gene product, β-galactosidase, in NIH/3T3 mouse fibroblasts. Stable transfectants were generated to facilitate studies of gene regulation by cell growth state. The expression of FN-*lacZ* constructs increased approximately twofold when cultures attained confluence, relative to total protein. The magnitude of this increase correlates well with that observed for FN mRNA levels and protein synthesis rate. Fragments containing 4.9, 0.9, or 0.3 kbp upstream of the transcription start site are equally responsive to cell density and/or cell contact. Deletion of a cAMP-responsive element enhanced the response, suggesting a negative role for this sequence motif and demonstrating that the FN gene is regulated by cell density at the transcriptional level.The effect of high cell density is apparently different from decreased growth rate, as incubation with low serum did not result in increased expression of the *lacZ* reporter. Finally, conditioned medium from dense cells did not enhance reporter gene expression in sparse cells, suggesting that the density signal is not transmitted via a soluble factor.

Key words: fibronectin, gene regulation, cell growth, *lacZ*, NIH/3T3

The physical environment of a cell includes cell-cell and cell–extracellular matrix (ECM) interactions in addition to soluble factors. The ECM that borders or surrounds many cells is complex, composed of proteins and proteoglycans. ECM deposition changes dramatically during normal embryonic development, and evidence has accumulated that matrix composition can strongly influence cell differentiation [Adams and Watt, 1993]. Thus, a complicated interplay most likely exists between a cell and the surrounding ECM.

One group of ECM molecules implicated in cell differentiation and cell motility is the fibronectins (FNs). Fibronectins are a family of large dimeric glycoproteins with numerous adhesive activities that permit interactions with cells and macromolecules [Hynes, 1990; Mosher, 1989]. Fibronectins are required for proper formation of mesodermal structures; mouse embryos that are genetically null for FNs exhibit severe defects consistent with aberrant cell migration [George et al., 1993]. Amphibians appear to require FN for proper gastrulation [Boucaut et al., 1984; DeSimone, 1994; Smith et al., 1990]. Striking changes in ECM also occur as a consequence of tissue injury. The initial phase of wound healing involves the rapid assembly of a fibrin clot, which contains plasma FN and other circulating and platelet-derived ECM molecules [Gailit and Clark, 1994]. Subsequently, additional isoforms of FN are produced by cells that migrate into the wound bed [Brown et al., 1993; ffrench-Constant et al., 1989]. Still later, this provisional matrix is replaced by one that is collagen-rich [Kurkinen et al., 1980; Whitby and Ferguson, 1991]. These findings are consistent with a role for FNs early in the process of matrix assembly. Similar processes appear to occur during experimental models of liver and lung fibrosis [Martinez-Hernandez, 1984; McDonald, 1989].

The production of fibronectin by cells in culture has been studied extensively. Fibronectin expression can be modulated by numerous cytokines and growth factors [reviewed in Dean, 1989; Gailit and Clark, 1994; Thiery and Boyer, 1992]. Serum and epidermal growth factor (EGF) tend to elevate FN expression, primarily at the

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transcriptional level [Blatti et al., 1988; Dean et al., 1990; Ryseck et al., 1989]. FN levels can also be modulated by cell density; specifically, the rate of FN synthesis in sparse, growing cultures of Nil8 hamster fibroblasts was relatively low but increased dramatically as the cells approached confluence [Senger et al., 1983]. Similarly, FN mRNA levels were elevated in confluent dermal fibroblasts and smooth muscle cells in comparison with levels in sparse, growing cells [Brown et al., 1991; Choi et al., 1992]. In other studies, FN mRNA levels were elevated by rendering cells nonadherent [Dike and Farmer, 1988]. Thus, the FN gene responds to complex environmental cues.

We have begun to identify the rat FN DNA sequences that control the gene's regulation in vitro and in vivo. Initial studies have focused on establishing whether the regulation of FN expression by cell growth could be conferred onto a reporter gene. To this end, a *lacZ* reporter gene has been placed under the control of DNA sequences derived from the rat FN gene [Patel et al., 1987]. We show that fragments of rat FN DNA containing as little as 0.3 kbp of 5′ flanking sequence and 136 bp of 5′ untranslated sequence confer density-dependent reporter gene expression, which closely parallels the increase observed for FN synthesis and FN mRNA levels in these cells. Furthermore, our results suggest that the cessation of growth associated with contact inhibition is not equivalent to that induced by serum withdrawal, and the signal is not transmitted via soluble factors.

METHODS

Plasmids

The starting construct, pZ2, contains the *lacZ* coding region flanked by several restriction sites [McInnis et al., 1995], permitting insertion of sequences both 5′ and 3′ of *lacZ*. Plasmid pZ2SH was generated by blunt-ended ligation of a SacII-HindIII fragment from the rat FN gene [Patel et al., 1987] to a unique SphI site downstream of *lacZ;* this fragment contains 515 nucleotides of 3′ untranslated sequence and 204 nucleotides beyond the site of polyA addition. Plasmid pZ2GH was generated by insertion of a growth hormone gene SmaI-EcoRI fragment derived from pLENX (see below) at the SphI site; this fragment contains 100 nucleotides of 3′ untranslated sequence and 527 nucleotides of sequence beyond the site of polyadenylation.

A 1.0 kbp StuI-PstI fragment was isolated as a KpnI-XbaI fragment from a subclone of the rat FN gene [Patel et al., 1987]. This fragment extends from −880 to +136 relative to the transcription start site (+1) and was subcloned into the KpnI-XbaI sites upstream of *lacZ* in pZ2SH, generating plasmid pFNZ0.9. An AccI-PstI fragment (−318 to +136) was similarly subcloned into pZ2SH, generating plasmid pFNZ0.3. Plasmid pFNZ0.3A was generated by linearization of pFNZ0.3 at a unique AatII site (−155), treatment with the Klenow fragment of DNA polymerase, and religation. Sequencing of this plasmid revealed a deletion of 18 bp, including most of the cAMP response element. An approximately 5.0 kilobase pair (kbp) PstI fragment was excised from λrFN9 [Patel et al., 1987]. This fragment includes the 1.0 kbp piece described above, with additional 5′ flanking sequence. The 5.0 kbp fragment was inserted into pZ2SH as above, generating pFNZ4.9. Thus, all plasmids should produce *lacZ* transcripts containing 136 nucleotides of 5′ and 513 nucleotides of 3′ untranslated sequence derived from rat FN (the complete regions are 207 and 691 nucleotides, respectively). The 4.9 kbp PstI fragment was inserted into pZ2GH, generating pFNZ4.9GH.

Plasmid pLENXZ was generated by excising the *lacZ* cassette of pZ2 and inserting it into pLENX, a modified version of pLEN, which contains the mouse metallothionein promoter, the SV40 enhancer, and the 3′ end of the human growth hormone gene [Guan et al., 1990; Neufeld et al., 1988]. Details of the structure of this plasmid have been described elsewhere [McInnis et al., 1995].

Cell Culture and Transfections

NIH/3T3 mouse embryo fibroblasts (gift of R. Frackelton, Roger Williams Medical Center, Providence, RI) were maintained in Dulbecco's MEM (D-MEM) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin. For selection of stable transfectants, medium was supplemented with 0.5–0.7 mg/ml geneticin (G418) (Gibco/BRL, Gaithersburg, MD). Following selection, cultures were maintained in 0.25 mg/ml G418.

Both stable and transient transfections were performed as follows. NIH/3T3 cells were plated 24 h prior to transfection at 2.5×10^5 cells per 35 mm diameter well. DNAs (3 μg) were mixed with 10 μl of Lipofectamine reagent (Gibco/

BRL) in serum-free medium (SFM). Cells were incubated with the DNA-lipid mix at 37°C for 5–6 h; then the medium was replaced with fresh D-MEM/10% FCS. Transfected cultures were incubated for 3 days and then either assayed for β-galactosidase activity (transient transfection) or trypsinized and seeded into two 10 cm diameter dishes in the presence of G418 (stable transfection). After 10–14 days of selection, one plate was stained for *lacZ* expression, and the other plate was subcultured. Clonal isolates were derived by limiting dilution.

For experiments evaluating the influence of cell density, subconfluent cells were trypsinized and seeded in a twofold serial dilution into 35 mm diameter wells. Duplicate wells were plated for each condition. Cells were incubated until the densest wells were confluent with few mitotic cells visible. The area of the dish covered by cells in the other wells was estimated visually, and this fraction is defined as percent confluence. Experiments were performed a minimum of three times.

Assays for β-Galactosidase Activity

The quantitative solution assay for β-galactosidase activity was as described [Norton and Coffin, 1985]. Briefly, cells were washed in Dulbecco's phosphate-buffered saline (PBS) and lysed in this buffer containing 0.1% sodium dodecyl sulfate (SDS). Next, 0.8 ml of assay buffer was added (33 mM NaH_2PO_4, 66 mM Na_2HPO_4, 2 mM $MgSO_4$, 0.1 mM $MnCl_2$, and 40 mM β-mercaptoethanol), followed by 0.2 ml of the colorimetric substrate *o*-nitrophenyl-β-D-galactoside (ONPG) (freshly made at 4 mg/ml in assay buffer). Following incubation at 37°C, reactions were stopped by addition of 0.5 ml 1.0 M Na_2CO_3. Units of enzyme activity are defined as A_{420} multiplied by 380, divided by incubation time (minutes). For transient transfections, values were corrected for differences in plasmid size by expressing the activity as units per picomole of DNA transfected.

To assess the percentage of stably transfected cells that express the *lacZ* reporter gene, cells were fixed in 3.7% formaldehyde in PBS and stained with the colorimetric substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) essentially as described [Sanes et al., 1986]. Quantitation of β-galactosidase was performed using the solution assay described above, but a portion of each lysate was reserved prior to ONPG addition for protein determination (BioRad, Richmond, CA). The precise volume used varied to ensure that samples fell within the linear range of the assays.

Metabolic Labeling, Immunoprecipitation, and SDS-Polyacrylamide Gel Electrophoresis

Cells were plated at 1×10^5 or 8×10^5 per 35 mm diameter well. After 48 h, the former wells were <50% confluent, whereas the latter were confluent, with few mitoses visible. Monolayers were washed with D-MEM lacking methionine and cystine, and then 0.5 ml of this medium containing 100 μCi [^{35}S]-amino acids (New England Nuclear, Boston, MA) was added. After incubation at 37°C for 15 min, cells were washed with PBS and stored at −80°C. Thawed monolayers were lysed in 0.5 ml RIPA buffer (0.5% sodium deoxycholate, 0.5% NP-40, 20 mM Tris-HCl, pH 8.3, 50 mM NaCl, 2.0 mM PMSF, and 2.0 mM EDTA). After brief centrifugation, aliquots of each supernatant were precipitated with trichloroacetic acid (TCA). Equal numbers of TCA-precipitable counts were incubated with 10 μl polyclonal antibody directed against mouse fibronectin (Telios, San Diego, CA) for >1 h at 4°C; then proteinA-agarose beads (30 μl) were added. After an additional hour at 4°C, beads were washed three times with RIPA buffer, resuspended in sample buffer (4.0% SDS, 0.2 M DTT, 0.16 M Tris-HCl, pH 6.8, 4.0 mM EDTA, 20% glycerol, and 0.1% bromphenol blue) and boiled. Electrophoresis was through 6.5% polyacrylamide separating gels. Gels were fixed in 10% methanol/7% acetic acid, treated with Enlightning (New England Nuclear), and then dried. The gel was exposed to a PhosphoImage screen (Molecular Dynamics, Sunnyvale, CA) and then to X-ray film. The entire experiment was repeated twice (four pairs of wells).

RNA Isolation

Cells were plated at 4.8×10^6 or 6.0×10^5 per 85 mm diameter dish; this seeding regimen is equivalent to that used for the metabolic labelling of FN. After 48 h, the former were confluent, and the latter were <50% confluent. Cells were washed with PBS, then Trizol reagent (Gibco/BRL) was added (4 ml/plate), and the lysate was collected. Chloroform (0.2 ml $CHCl_3$/ml Trizol) was added, gently mixed for 5 min, and spun for 30 min at 3,000 rpm. The supernatant was recovered and precipitated with an equal volume of 2-propanol. After centrifugation at 10,000 rpm for 30 min, the RNA pellet was washed with 70% ethanol, dried, and resuspended in 20–100 μl of water. Concentration

was determined spectrophotometrically and confirmed by agarose gel electrophoresis. Several pairs of samples were analyzed by either RNase protection or dot blot (not shown).

RNA Probe Preparation and RNase Protection Assay

A 480 bp EcoRI-HindIII fragment of mouse fibronectin cDNA was subcloned into pBS- (Stratagene, La Jolla, CA); this fragment contains 27 bp of alternative exon EIIIB, with the remaining sequences present in all FN mRNAs [P. Norton, unpublished results]. RNase protection probes were generated from EcoRI-cleaved template DNA by in vitro transcription in the presence of α-[^{32}P]-UTP and T3 RNA polymerase (Stratagene). The radiolabeled antisense transcript was purified by denaturing gel electrophoresis and eluted at 37°C overnight in 0.3 M sodium acetate, 0.5% SDS. The probe was precipitated with ethanol and resuspended in 100 μl of water.

RNase protection was performed using modifications of published procedures [Haines and Gillespie, 1992; Thompson and Gillespie, 1987]. Typically, 1–2 μl of probe ($>10^5$ cpm/μl) was mixed with 5–10 μl total cellular RNA and 40–45 μl 6 M guanidinium isothiocyanate. Samples were heated to 65°C for 15 min and incubated overnight at 45°C. Each reaction was expanded by the addition of 500 μl of 0.75× SSC containing RNaseA (10 μg) and RNase T1 (50 units), and samples were incubated at 45°C for 30 min. RNase-resistant fragments were precipitated by the addition of 1.0 ml ethanol containing 2% diethylpyrocarbonate and 5 μg carrier yeast tRNA. The RNA was collected by microcentrifugation for 20–30 min and resuspended in formamide loading buffer (90% formamide, 0.1% each bromphenol blue and xylene cyanol, 1 mM EDTA). Samples were heated for 2 min at 90°C and then applied to a 6% denaturing gel. The dried gel was exposed to a PhosphoImage screen (Molecular Dynamics) and then to X-ray film.

RESULTS

FN Sequences Direct Expression of the *lacZ* Reporter Gene in Transient and Stable Transfection Assays

Several *lacZ*-containing reporter plasmids were constructed so as to contain sequences from the 5′ and/or 3′ end of the rat FN gene; these are diagrammed in Figure 1. NIH/3T3 cells were transfected with equal amounts of the above DNAs as well as promoterless versions (pZ2SH and pZ2GH). Three days posttransfection, cultures were lysed and assayed for β-galactosidase activity. Figure 1 indicates the units of β-galactosidase produced per picomole of *lacZ* DNA transfected. Plasmid pLENXZ, with *lacZ* under the control of the metallothionein promoter combined with the SV40 enhancer, was expressed at a high level [McInnis et al., 1995]. The FN promoter-driven plasmids were expressed at lower levels than pLENXZ. The largest plasmid, pFNZ4.9, was somewhat more active than pFNZ0.9 or pFNZ0.3. Deletion of the cAMP response element (CRE) [Dean, 1989], as in plasmid pFNZ0.3A, reduced β-galactosidase levels to background. However, appreciable background expression of the promoterless constructs was observed, perhaps due to readthrough from fortuitous promoter elements in the vector (see below). We conclude that the rat FN promoter sequences are functional. The higher activity of pFNZ4.9 relative to pFNZ4.9GH suggests that FN 3′ sequences also influence reporter gene expression.

We reasoned that studies of the influence of culture conditions would be facilitated by the preparation of stably transfected cells. Thus, NIH/3T3 cells were cotransfected with pSV2neo and pFNZ4.9. Three days posttransfection, cells were replated at low density and placed under G418 selection. The number of drug resistant cells that also acquired and expressed pFNZ4.9 was estimated by staining portions of the cultures with X-gal. Approximately 15% of all cells stained blue, with most of these cells staining intensely (data not shown). In contrast, stable pools of pZ2SH or pZ2GH transfectants exhibited few, lightly stained cells and little β-galactosidase activity by solution assay (data not shown). Thus, high background activity of the promoterless plasmids (Fig. 1) appears to be a consequence of the transient nature of the experiment.

FN Protein and mRNA Levels Are Influenced by Cell Density

FN protein and/or RNA levels have been shown to vary with cell density in several cell types [Brown et al., 1991; Choi et al., 1992; Raghow et al., 1987; Senger et al., 1983]. Metabolic labeling was performed to determine the extent of modulation of FN synthesis by cell density in FNZ4.9-transfected cells (FNZ4.9 cells). Sparse (<50% confluent) or dense (100% confluent) cultures were pulse-labeled with ^{35}S-

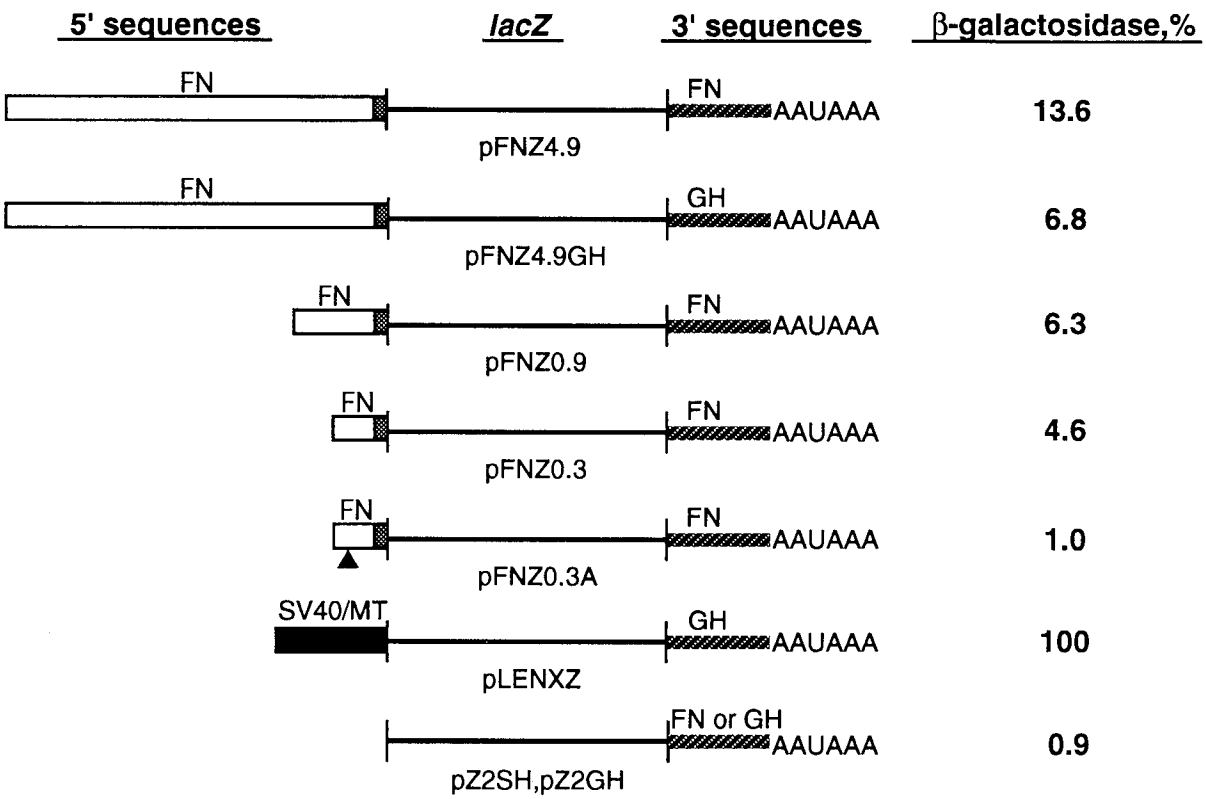

Fig. 1. Structure of FN-*lacZ* plasmids and activity in a transient transfection assay. Plasmids containing a *lacZ* reporter are diagrammed; the construction of these is detailed in Methods. Promoter sequences are denoted by open bars (FN) or closed bars (SV40/metallothionein) (see Methods) and the 5′ untranslated region by stippled bars. The *lacZ* reporter is indicated by a line, and 3′ untranslated regions and polyA addition sites (from either the FN gene or the human growth hormone gene) are indicated by a hatched bar. Also shown is the level of β-galactosidase produced by each reporter relative to reference plasmid pLENXZ. Values reflect the units of β-galactosidase activity per picomole of transfected plasmid DNA, to adjust for the significant difference in size of the constructs. Each value represents the average of at least three transfections.

amino acids. Aliquots of cell lysates containing equal numbers of TCA-precipitable counts were immunoprecipitated with a polyclonal antibody directed against mouse fibronectin. PhosphorImager quantitation of the results shown in Figure 2 demonstrated a twofold increase in the rate of FN synthesis in the dense cells relative to the sparse cells (averages for each pair of lanes are 4.1×10^5 vs. 1.9×10^5 integrated volumes). Thus, FN levels are regulated by cell density and/or cell contact in NIH/3T3 cells; for convenience we will use the the term *cell density*.

Total RNA was isolated from pFNZ4.9 cells that were either sparse (<50% confluent) or dense (100% confluent). The amount of RNA used for each sample was normalized to ribosomal RNA, as determined by densitometric measurement of stained agarose gels (not shown). FN mRNA levels were measured by RNase protection using an RNA probe transcribed from a mouse FN cDNA. Quantitation of the results shown in Figure 3 by PhosphorImager analysis indicate that dense cultures contained approximately twice as much FN mRNA as sparse cells (levels of the lower band in lanes 5–8 are on average 2.0 times higher than the comparable band in lanes 1–4). Thus, density-dependent regulation of FN protein synthesis can be accounted for by the increase in steady-state FN mRNA levels, as normalized to ribosomal RNA.

The possibility that cell density could alter FN alternative splicing was also considered. However, the RNase protection assay indicated that the level of inclusion of alternative exon EIIIB was unchanged by cell density (a ratio of twice the amount of B− as B+ mRNA).

FN Sequences Confer Density-Dependent Expression on a *lacZ* Indicator Gene

To determine whether the *lacZ* reporter gene could respond to cell density, FNZ4.9 cells were seeded at different densities in a twofold dilution series (in duplicate). Cultures were allowed to

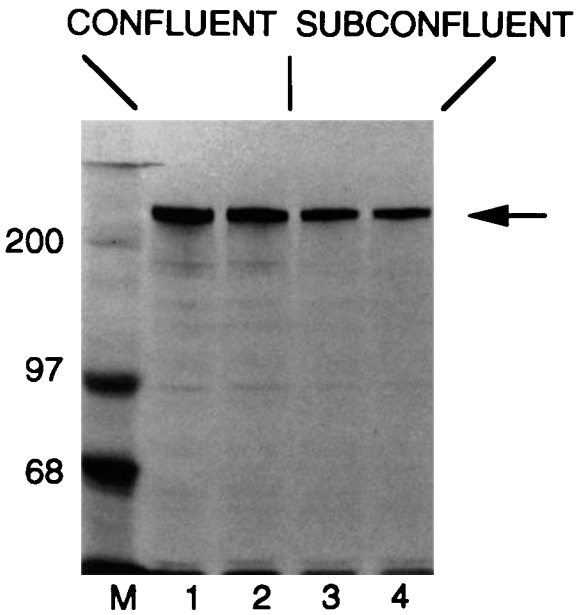

Fig. 2. Rates of FN synthesis in confluent versus subconfluent cells. Duplicates of confluent (*lanes 1, 2*) or sparse (subconfluent) (*lanes 3, 4*) cultures were metabolically labeled with [^{35}S]-amino acids for 15 min. Cells were lysed, and equivalent numbers of TCA-precipitable counts were incubated with antiserum to FN and proteinA-agarose. Immunoprecipitates were washed, and denatured, reduced samples were subjected to SDS-PAGE. The FN band is indicated by the *arrow*. *Lane M*: [^{14}C]-labelled protein standards (kDa).

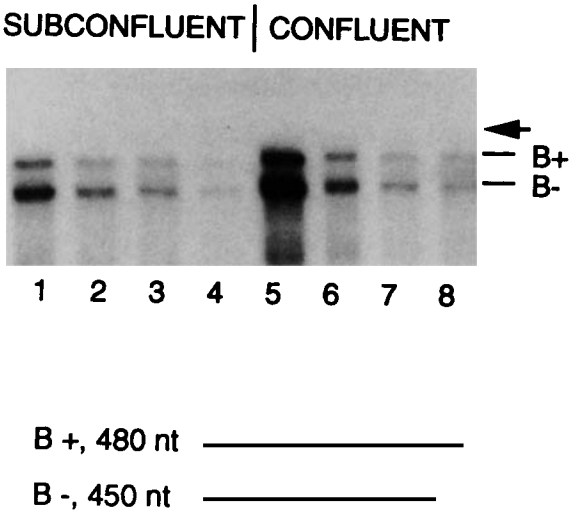

Fig. 3. FN mRNA levels in confluent vs. subconfluent cells. RNA was isolated from subconfluent and confluent cultures, and decreasing amounts of each sample were incubated with a constant amount of uniformly labeled mouse FN antisense RNA probe. Following RNase digestion, specific hybrids were analyzed by denaturing polyacrylamide gel electrophoresis. The position of undigested probe is indicated by the *arrow;* the two protected fragments corresponding to the EIIIB+ and EIIIB− forms of FN are indicated (B+ and B−) and diagrammed below. *Lanes 1* and *5* contained 5 μg total RNA; the remaining lanes represent twofold serial dilutions from this initial concentration. Note that lane 5 appears to have been slightly overloaded.

grow until the most densely seeded wells had reached complete confluence, and then cells were trypsinized and counted; duplicate values were averaged. As shown in Figure 4A, cells plated at 1.6×10^6 per well underwent less than one population doubling over the 2 day incubation. In contrast, the less densely seeded wells expanded by 2.5-fold. The degree of confluence of less densely seeded wells was estimated visually to facilitate comparisons between experiments. Next, FNZ4.9 cells were seeded at the same dilutions and incubated for 2 days. Cells were lysed in situ and β-galactosidase assays performed; values from duplicate wells were averaged. The values for the sparsest wells were set as unity, and the other wells were normalized to this value (Fig. 4B). Assuming the growth rate illustrated in Figure 4A, the expected increase in β-galactosidase activity with increased plating density is plotted for comparison. Thus, as the increase in enzyme activity exceeds the increase expected from cell division, it appeared that the expression of the reporter gene responded to cell density.

Because cell number determination was not well adapted for use with the enzyme assay protocol, we chose to normalize reporter activity to total protein content, as this is comparable to the normalization of FN mRNA to total RNA. Cells were seeded in a twofold serial dilution and cultured until the densest wells reached confluence. Cells in individual wells were lysed, and portions of each lysate were assayed for either β-galactosidase activity or total protein. Figure 4C shows the results of such an experiment; values from duplicate wells were averaged. Values from the most dense wells were set to 100%. The ratio of β-galactosidase to total protein was approximately twofold higher in dense cultures relative to the sparse wells. (Cells at intermediate density tended to exhibit an intermediate ratio of β-galactosidase to total protein.) Thus, the *lacZ* indicator gene closely reflected the behavior of the endogenous FN gene in these cells (Figs. 2, 3).

A second pool of pFNZ4.9 transfectants exhibited identical density-dependent *lacZ* expression (data not shown). In addition, individual *lacZ*-expressing subclones were derived by limiting dilution. The three clones examined on average behaved similarly to the polyclonal population (Table I). Thus, density dependence is largely independent of the integration site of the transfected DNA.

Density Dependence Is Conferred by <1.0 kbp of 5′ FN Sequence, With no Requirement for 3′ Untranslated Sequence

Increased steady-state RNA levels could be due to either an increase in transcription initiation or to increased mRNA stability. To determine whether FN 3′ untranslated sequences are required for density dependence, NIH/3T3 cells were cotransfected with SV2neo and pFNZ4.9GH and selected for G418 resistance. The latter plasmid has the 3′ end of the human growth hormone gene substituted for the 3′ FN sequences (Fig. 1). X-gal staining indicated that the pool contained approximately 10% *lacZ*-positive cells (data not shown). Cells from this pool were serially diluted and analyzed for β-galactosidase activity and total protein as for FNZ4.9 cells. A similar confluence-dependent change in β-galactosidase levels relative to total protein levels was observed (Fig. 5A), indicating that FN 3′ sequences are not required for regulation.

Additional experiments were performed to further delineate the upstream FN sequences that

TABLE I. Density-Dependent β-Galactosidase Activity of Subcloned FNZ4.9 Transfectants

Clone	Activity at 100% confluence (u/μg)	Activity at 25–50% confluence (u/μg)	Fold stimulation
I	1.4	.95	1.5
1	.88	.42	2.1
5	2.25	1.0	2.25
22	2.1	1.4	1.5
Average			1.8

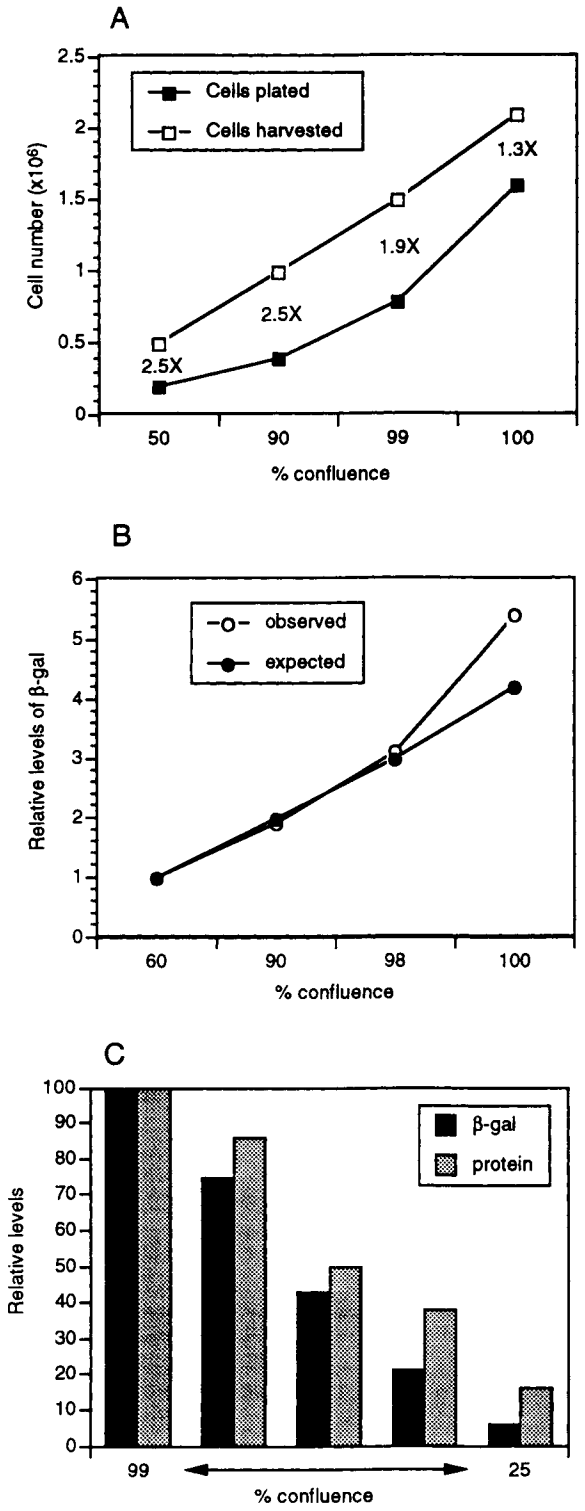

Fig. 4. Influence of cell density on expression of the *lacZ* reporter pFNZ4.9. **A:** FNZ4.9 cells were plated in duplicate at the concentrations indicated by the filled squares. After 48 h in culture, cells were trypsinized and collected by centrifugation, and a portion was counted. The number of cells present is indicated by the open squares; values from duplicate wells were averaged. The numbers indicate the fold increase in cell number. **B:** Stably transfected FNZ4.9 cells were plated in duplicate at the same cell numbers as in A (0.2, 0.4, 0.8, or 1.6 × 10^6/well). Two days later culture confluence was estimated visually, and then each well was assayed for β-galactosidase activity (open circles). The value for the lowest point tested was set to unity and the other values adjusted in relation to that point. The closed circles represent the projected increase in enzyme activity if enzyme activity was strictly a function of cell number, using growth rates derived from A. **C:** FNZ4.9 cells were plated in a twofold dilution series in duplicate. After 3 days, cells were lysed and portions of each sample assayed for β-galactosidase activity (black bars) and total protein (stippled bars). The highest data points for each assay have been adjusted to 100% and plotted against the visually estimated level of cell confluence. Each bar represents the average of duplicate samples; duplicate values varied less than 10% for enzyme activity and less than 20% for protein concentration.

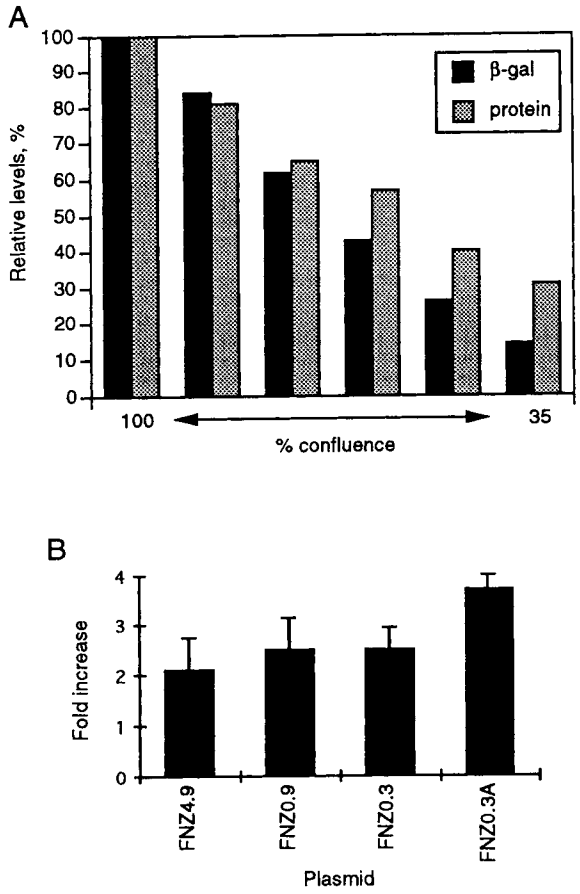

Fig. 5. Influence of cell density on FN-*lacZ* reporter plasmids with reduced amounts of FN sequences. **A:** FNZ4.9GH cells were plated in twofold serial dilution and assayed 4 days later for β-galactosidase activity (black bars) and total protein (stippled bars), as described in the legend to Fig. 4. **B:** FNZ4.9, 0.9, 0.3, and 0.3A cells were plated in duplicate in twofold serial dilution and assayed 2–3 days later for β-galactosidase activity and total protein. β-galactosidase and protein levels in sparse cells were set at 1.0. The relative increase for β-galactosidase and protein in dense cells was calculated, then the relative increase of β-galactosidase was divided by the relative increase in protein. The resulting value is defined as the fold increase and is a measure of the level of density-dependent induction of the *lacZ* reporter at high cell density. Data was taken from three experiments and the fold increase values averaged; the averages for each plasmid are plotted (bars) along with the standard errors (lines).

render the *lacZ* reporter gene sensitive to cell density. NIH/3T3 cells were cotransfected with SV2neo and pFNZ0.9, pFNZ0.3 and pFNZ0.3A and subjected to G418 selection. Analysis of duplicate pools for each plasmid revealed that typically 5–15% of the cells also contained the *lacZ* reporter, as assessed by X-gal staining, making precise comparisons between the plasmids difficult. However, the solution assays suggest an ordering of FNZ4.9 > FNZ0.9 = FNZ0.3 > FNZ0.3A, consistent with the transient transfection data (Fig. 1). FNZ0.3A cells had β-galactosidase levels about half those of FNZ0.3, suggesting that the CRE mutation is not as deleterious to promoter function when stably integrated as in the transient experiments.

The individual transfected pools were plated at high and low cell density, cultured for 2–3 days, and then assayed for β-galactosidase activity and total protein. All plasmids responded to cell density (Fig. 5B). Indeed, FNZ0.3A cells exhibited an enhanced response to density; β-galactosidase levels were induced nearly fourfold in dense relative to sparse cells in comparison to total protein, in contrast to the approximately two- or 2.5-fold induction seen for the other plasmids. Thus, only 318 bp of 5′ flanking DNA from the FN gene is sufficient to confer density dependence.

Cells Rendered Quiescent by Serum Withdrawal Do Not Show Increased Expression of the Indicator Gene

The increased expression of *lacZ* with high cell density might be due to either increased cell-cell contact or to the reduced growth rate. To differentiate between these two possibilities, FNZ4.9 cells were seeded sparsely in D-MEM supplemented with 10% FCS and incubated for 2 days. Serum was decreased to 0.5% to reduce cell growth, and cultures were assayed 18 h later for β-galactosidase activity. Serum-depleted cultures did not exhibit increased β-galactosidase levels relative to controls (Table II). In another experiment, it was determined that although serum decrease slightly reduced cell number, untreated control wells had an average of 28 U β-galactosidase/10^6 cells, whereas the serum-depleted cells had 23 U/10^6 cells (data not shown). Thus, lower serum resulted in a slight repression of the reporter gene. In contrast, addition of medium containing 10% FCS cultures resulted in an increase of *lacZ* expression (Table II). Both sparse and dense cultures responded to serum by increasing reporter expression (data not shown), suggesting that the density and serum responses are separable. Thus, the stimulation of *lacZ* expression at confluence apparently is due to altered cell-cell contacts or cell shape, not reduced cell growth rate.

The Density Signal Is Not Transmitted via a Soluble Factor

We wished to test whether cells increase FN synthesis in response to depletion of serum FN from the medium during growth. Cells were

TABLE II. Effect of Serum Addition or Withdrawal on Expression of *lacZ* in FNZ4.9 Transfectants

Treatment[a]	β-gal, U[b]
18 h in original medium	7.8
18 h in D-MEM plus 0.5% FCS	5.6
18 h in fresh D-MEM plus 10% FCS	10.4

[a]All cells were held for 48 h in D-MEM plus 10% FCS prior to treatment.
[b]A similar fraction of each lysate was assayed for β-galactosidase activity.

plated in the presence of medium containing either 10% complete serum or 10% FN-depleted serum (serum was passed over a gelatin-agarose column and the flow-through collected). No difference in the level of *lacZ* expression was observed following FN depletion from the serum (not shown). Thus, the signal to synthesize more FN is probably not the level of soluble FN.

A more direct test of the hypothesis that density information can be transmitted via soluble factors was performed. FNZ0.9 cells were plated in triplicate at either 1×10^5 or 8×10^5 cells per 35 mm well. After incubation for 24 or 48 h, when the denser cells were nearly confluent, conditioned medium was exchanged (sparse to dense and dense to sparse). Control wells were left untreated. After incubation for an additional 24 h, the cells were assayed for β-galactosidase levels. The sparse cells that received medium conditioned by the dense cells did not exhibit increased *lacZ* expression (average enzyme activity of .72 vs. .99 units for controls). Similarly, there was no repression of *lacZ* expression in dense cells by exposure to medium conditioned by sparse cells (average enzyme activity of 7.4 vs. 6.7 units for controls). Thus, cell density information does not appear to be transmitted as a soluble factor and is probably signaled by alterations in cell-cell contacts, cell shape, or motility.

DISCUSSION

A *lacZ* reporter gene was placed under the control of sequences from the rat FN gene and the resulting plasmids evaluated for *lacZ* expression in transfection assays. The data presented demonstrate the activity of FN DNA fragments in transiently transfected mouse NIH/3T3 cells; we have obtained similar results in rat skin fibroblasts (data not shown). Activity of the reporter gene has also been evaluated in stably transfected cells. Expression of the reporter is sensitive to cell density, similar to the endogenous FN gene, and this regulation is not mediated by soluble factors.

Activity of the Rat Fibronectin Promoter Region

The activity of the FN fragments is generally consistent with reports of the rat promoter activity in rat and mouse fibroblasts and the human promoter in several cell types including NIH/3T3 [Dean et al., 1988, 1989; Miao et al., 1993; Muro et al., 1992; Nakajima et al., 1992]. The difference in the activity of pFNZ4.9 and pFNZ0.9 is in agreement with the presence of a fibroblast enhancer element at approximately −2.0 kbp, although we do not observe the same magnitude of induction [Sporn and Schwarzbauer, 1995]. Similarly, the reduced activity of pFNZ0.4A in the transient assay is consistent with the behavior of similar CRE site mutations in mouse fibroblasts [Dean et al., 1989; Miao et al., 1993].

Many agents modulate FN levels by altering the rate of transcription initiation. The human FN promoter confers sensitivity to serum and forskolin onto a CAT reporter gene by via the CRE. The serum responsiveness of pFNZ4.9 in NIH/3T3 cells agrees with the behavior of the human gene in these cells [Dean et al., 1989] and with the known sensitivity of the endogenous mouse gene [Ryseck et al., 1989]. The effect reported in Table II is more modest than reported for the human FN promoter [Dean et al., 1990], but the latter experiments were performed using 20% FCS rather than 10%. Our data and that of others [Williams and Allen-Hoffmann, 1990] indicate that serum can further increase FN synthesis in dense cells. However, our findings contrast with reports that the rat FN promoter is repressed by serum [Hara et al., 1988; Nakajima et al., 1992]. The latter experiments were done in the rat fibroblast line 3Y1 and may be specific for those cells.

Regulation of FN and Reporter Gene Expression by Cell Density

We have demonstrated that mouse FN protein and mRNA levels are modulated by cell density in mouse NIH/3T3 fibroblasts. However, the magnitude of the density response (twofold) is considerably less than that observed for Nil/8 hamster embryo fibroblasts [Senger et al., 1983]. Other workers examining various fibroblasts or smooth muscle cells have reported smaller increases in FN and FN mRNA with increased confluence [Brown et al., 1991; Choi

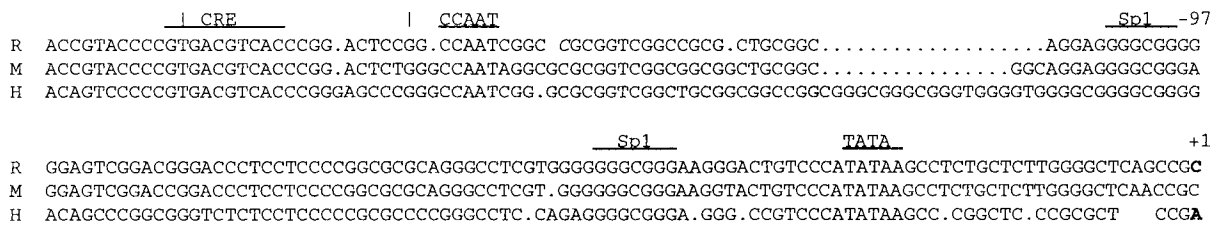

Fig. 6. Comparison of rat, mouse, and human FN sequences surrounding the transcription initiation site. The sequences shown were retrieved from the Genbank DNA database. The rat sequence [Nakajima et al., 1992; Patel et al., 1987] has an additional base at position −130 (italic) relative to the published sequence, based on additional sequencing and restriction enzyme cleavage data (the additional C creates a SacII site). The human (H) [Dean et al., 1987] and rat (R) sequences were aligned by Bestfit (Genetics Computer Group, Madison, WI); the mouse (M) [Polly and Nicholson, 1993] sequence was aligned manually with the closely related rat sequence. The sites of transcription initiation for the human and rat genes are in bold. Various transcriptional control signals [Dean, 1989; Muro et al., 1992; Nakajima et al., 1992; Patel et al., 1987] are overlined; these are the CRE (−160 to −151), CCAAT (−139 to −135), Sp1 (−104 to −99 and −50 to −44), and TATA elements (−29 to −25). The base pairs deleted in pFNZ0.3A are denoted by the vertical bars.

et al., 1992; Hatamochi et al., 1989; Liau and Chan, 1989; Raghow et al., 1987]. Thus, while the magnitude of the cell density response might vary from one cell type to another, a general correlation exists between increased cell density and increased FN expression. It is also possible that further upregulation of the FN gene would be observed if the cells were held longer at confluence [Owen et al., 1990], but we have not tested this possibility.

The ability to confer density-dependent regulation onto a reporter gene allows us to investigate the sequence elements involved. As little as 318 bp of sequence 5′ to the transcription start site was sufficient to confer density-dependent gene regulation. A parsimonious assumption is that both rat and mouse sequences possess the elements needed to confer sensitivity to cell density, as both the endogenous gene and the rat FN/*lacZ* reporter gene exhibited the same twofold response. Figure 6 shows an alignment of rat, mouse, and human FN promoter sequences. The mouse and rat sequences are highly conserved, with approximately 95% identity. The CRE and CCAAT motifs are conserved between the three mammalian sequences (Fig. 6), as are the TATA box and two SP1-type motifs. While cAMP has been reported to increase FN gene expression in NIH/3T3 cells [Dean et al., 1989], it was found to repress FN in a granulosa cell line [Bernath et al., 1990]. Our data show that deletion of the CRE element enhances the response to cell density. Thus, this element may play a dual role in FN regulation, acting in a negative as well as a positive fashion, depending on the circumstances. The CCAAT and CRE elements synergize in the liver but not in other tissues [Miao et al., 1993; Muro et al., 1992; Srebow et al., 1993]. Such combinatorial regulation could allow great flexibility in the response of cells to complex environmental stimuli.

We have demonstrated that 3′ untranslated sequences are not involved in density-dependent FN gene expression (Fig. 5A). Furthermore, the enhanced response of pFNZ0.3A to cell density provides direct evidence that sequences outside of the transcribed region modulate FN gene expression. Thus, our results are most consistent with the hypothesis that cell density regulates FN gene expression via transcription initiation.

Possible Mechanisms and Implications of Increased FN Synthesis With Density

The mechanisms whereby complex environmental cues alter gene expression are incompletely understood. Other genes that respond to cell density include the RSV LTR [Lang et al., 1993] and several collagen genes [Brown et al., 1991; Hatamochi et al., 1989]. However, the FN gene presents an additional paradox. Serum stimulates both cell growth and increased FN expression; conversely, inhibition of cell growth (by high cell density, suspension culture [Dike and Farmer, 1988], or near senescence [Choi et al., 1992]) also results in increased FN expression. One possible resolution to this paradox is that the FN gene is sensitive to changes in cell shape. For convenience, we have been referring to the effects we observed as density-dependent. In reality, increasing cell confluence requires changes in cell shape and cell-cell vs. cell/substrate contacts. Interestingly, the increase in FN mRNA that occurs as normal human fibroblasts approach senescence [Burke and Danner, 1991; Choi et al., 1992; Kumazaki et al., 1991; Pagani et al., 1993] is paralleled by an increase in cell area [Kumazaki et al., 1991]. Thus, cell

morphology may be important in FN gene regulation, although the increased FN that we observe at confluence correlates with an apparent decrease in cell area. The ability of the reporter to respond to density should facilitate elucidation of these responses.

From the data presented, we conclude that cell density information was transmitted by direct cell-cell and or cell-ECM contacts and not through release of soluble factors. Cell shape information is likely conveyed to the nucleus by changes in the organization of the cytoskeleton. Treatment of cells with serum results in rapid reorganization of the actin cytoskeleton [Ridley and Hall, 1992]. Several members of the integrin family of receptors can transmit signals across the membrane and thus represent candidates for signaling cell density. Elevated intracellular pH follows ligand-mediated integrin activation [Schwartz, 1992], and intracellular pH is dependent upon cell density [Galkina et al., 1992]. Thus, cell-ECM interactions might be intertwined with regulation of ECM synthesis.

Finally, it is interesting to consider the in vivo relevance of cell density regulation. FN protein or mRNA can be observed to increase during mesenchymal cell condensation such as somite compaction [Perkinson and Norton, manuscript submitted] and chondrogenesis in the developing limb [Downie and Newman, 1994]. The attainment of confluence by cells in culture may reflect such in vivo situations. We are at present analyzing lines of mice that contain FNZ transgenes to begin to understand in vivo FN regulation.

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