Components of the Nuclear Signaling Cascade That Regulate Collagenase Gene Expression in Response to Integrin-derived Signals

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Abstract. We have shown previously that the expression of collagenase is upregulated in rabbit synovial fibroblasts cultured on a substrate of antibody to the α_5 chain of the $\alpha_5\beta_1$ integrin fibronectin receptor or on the 120-kD cell-binding chymotryptic fragment of plasma fibronectin, but remains at basal levels in cells plated on intact plasma fibronectin. We now have identified some of the components of a signaling pathway that couples the fibronectin receptor to the induction of collagenase transcription. We studied the control of collagenase gene expression in cells adhering to the 120-kD fragment of fibronectin, to anti-fibronectin receptor antibody, or to plasma fibronectin by transiently introducing promoter-reporter constructs into rabbit synovial fibroblasts before plating cells on these matrices. The constructs contained segments of the human collagenase promoter regulating transcription of chloramphenicol acyl transferase. Expression of constructs containing the −1200/−42-bp segment or the −139/−42-bp segment of the collagenase promoter inserted upstream from the reporter gene was induced to similar extents in cells plated on the 120-kD fragment of fibronectin or on anti-fibronectin receptor antibody, relative to that in fibroblasts plated on fibronectin. The expression of the construct containing the −66/−42-bp segment of the promoter was not regulated and was similar to that of the parent pBLCAT2 plasmid, suggesting that the −139/−67 region of the collagenase promoter, which contains PEA3- and AP1-binding sites, regulates the transcription of collagenase caused by integrin-derived signals. Expression of a reporter construct containing only the PEA3 and AP1 sites in the collagenase promoter (−90/−67) also increased in cells plated on the 120-kD fragment of fibronectin or on anti-fibronectin receptor antibody, relative to that in cells plated on fibronectin. Mutations in either the AP1 or PEA3 site of this minimal promoter abrogated its activity in cells plated on these inductive ligands. Expression of c-*fos* mRNA increased within 1 h of plating cells on the 120-kD fibronectin fragment or on anti-fibronectin receptor antibody, relative to that in cells plated on fibronectin. c-Fos protein accumulated in the nuclei of fibroblasts within 10 min of plating on the 120-kD fibronectin fragment. The increase in c-Fos was required for the increase in collagenase in cells plated on the 120-kD fibronectin fragment: incubation of cells with antisense, but not sense, c-*fos* oligonucleotides diminished both basal and induced expression of the −139/−42 collagenase promoter-reporter construct and decreased expression of the endogenous collagenase gene. Therefore, one of the pathways that transduce signals activated by the $\alpha_5\beta_1$ integrin fibronectin receptor propagates its response through promoters that contain AP1- and PEA3-responsive DNA sequences.

INTEGRIN receptors can play a role in cellular signaling cascades in different ways: integrin occupancy can modify or be modified by signals transduced by other ligand-receptor pairs or they may themselves initiate a signaling cascade. This inside-out and outside-in signaling implies a cooperative processing of information transduced by integrins (for review see Damsky and Werb, 1992; Hynes, 1992). Integrin occupancy can affect, or is a prerequisite for, several intracellular processes. For example, in activated platelets, the phosphorylation of a number of intracellular proteins requires the occupancy of $\alpha_{IIb}\beta_3$ receptor by ligand (Lipfert et al., 1992). Clustering of the $\alpha_5\beta_1$ fibronectin receptor (FNR)[1] with anti-FNR antibody regu-

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1. *Abbreviations used in this paper*: AP1, activator protein-1; CAT, chloramphenicol acyl transferase; CM, conditioned medium; ECM, extracellular matrix; FN, fibronectin; FNR, fibronectin receptor, FOS-OLIGO, synthetic oligonucleotide from c-*fos* sequences, in the sense orientation; LH, lactalbumin hydrolysate; MMP, matrix metalloproteinase; PEA3, polymavirus enhancer activator-3; RGD, Arg-Gly-Asp; RSF, rabbit synovial fibroblasts; SOF-OLIGO, synthetic oligonucleotide from c-*fos* sequences, in the antisense orientation; TPA, 12-O-tetradecanoyl phorbol-13-acetate; 120FN; the 120-kD chymotryptic fragment of human plasma fibronectin; uPA, urokinase-type plasminogen activator.

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lates the Na^+/H^+ antiporter, alkalinizing the cytoplasm (Ingber et al., 1990; Schwartz et al., 1991). Clustering of the FNR or adhesion to fibronectin (FN) also augments the lipid metabolism induced by PDGF (McNamee et al., 1993). Adhesion to FN also increases the expression of cyclin A (Guadagno et al., 1993).

Ultimately, the end point of signaling cascades is the regulation of gene expression and, thus, cell phenotype. Several studies have shown that this can occur in response to integrin-mediated binding to specific ligands. Integrins play a role in the regulation of terminal differentiation of myoblasts (Menko and Boettiger, 1987). The interaction of cells with FN or anti-integrin antibodies has been shown to inhibit the terminal differentiation of keratinocytes (Adams and Watt, 1989). Consequences of cell-ECM interactions have also been examined at the level of gene expression. Yamada et al. (1991) have shown that in T lymphocytes under some conditions, occupancy of the FNR induces the transcription factor AP1, leading to upregulation of interleukin-1. An enhancer element in the bovine casein gene, BCE-1, that regulates casein expression in mammary epithelial cells in response to concurrent signals from ECM receptors and prolactin receptors has also been described (Schmidhauser et al., 1990).

We have previously shown that rabbit synovial fibroblasts plated on FN fragments, anti-FNR antibody, or mixed substrates of FN and tenascin, but not on intact FN, induce the expression of three extracellular matrix (ECM)-degrading metalloproteinases (MMPs), collagenase, stromelysin, and the 92-kD gelatinase B (Werb et al., 1989; Tremble et al., 1993, 1994). The increase in collagenase mRNA and protein was detectable within 2–4 h of plating on FN fragments or on anti-FNR antibody, suggesting that the regulation of collagenase may be a direct response to a signaling cascade initiated by integrin receptors (Werb et al., 1989; Tremble et al., 1993, 1994). The human and rabbit genes for interstitial collagenase have been isolated (Angel et al., 1987*a*; Fini et al., 1987), and regulatory elements in the promoter that respond to growth factors, tumor promoters, and ultraviolet light have been identified (Radler-Pohl et al., 1993). In the present study we used constructs containing segments of the human collagenase promoter fused to a chloramphenicol acyl transferase (CAT) recorder gene to further define the transduction cascade regulating collagenase gene expression that is distal to integrin-derived signals in fibroblasts.

Materials and Methods

Cells and Cell Culture

Rabbit synovial fibroblasts (RSF), isolated as described previously (Aggeler et al., 1984), were cultured in DME (Cell Culture Facility, University of California, San Francisco) supplemented with 10% FBS (Hyclone, Denver, CO) and used between passages 2 and 10. Except where noted otherwise, RSF were subcultured 48 h before experimental procedures. Cells used in experiments were plated at subconfluent density on ECM-coated wells in DME supplemented with 0.2% lactalbumin hydrolysate (DME-LH), for example at a density of 250,000 cells per 1 cm^2 well. The plates were rotated to ensure even distribution of cells and incubated further at 37°C before analysis of MMP expression.

Antibodies

The anti-collagenase mAbs were characterized and used as described by Werb et al. (1989). The anti-stromelysin mAb (SL188.2) (Wilhelm et al., 1992) was a generous gift of Scott Wilhelm, Miles Research (West Haven, CT). The polyclonal anti-Fos antibody and matching peptide immunogen, purchased from CRB Biologics (Cambridge, England), were used in immunofluorescence studies. Adsorption of the anti-Fos antibody with the peptide immunogen was performed as described in the technical information supplied with the antibody. Antibodies to c-Fos (Ab-1) and c-Jun (Ab-2) that were used in immunoblotting were purchased from Oncogene Science (Uniondale, NY). The function-perturbing rat mAb, BIIG2, directed against the α_5 chain of the $\alpha_5\beta_1$ FNR, was used as described (Werb et al., 1989). The biotin- and horseradish peroxidase–conjugated secondary antibodies were purchased from Sigma Chem. Co. (St. Louis, MO), and Texas red–labeled streptavidin was purchased from Amersham Corp. (Arlington Heights, IL).

Preparation of Extracellular Matrix Substrates

Human plasma FN was purchased from Collaborative Research (Waltham, MA) or Boehringer Mannheim Biochemicals (Indianapolis, IN), reconstituted as directed by the manufacturer, and frozen at −70°C in single-use aliquots. The 120-kD chymotryptic fragment of human plasma FN (120FN) was purchased from Telios Pharmaceuticals (La Jolla, CA), reconstituted as directed, and stored in single-use aliquots at −70°C. ProNectinF, a poly-Arg-Gly-Asp (RGD) compound, was purchased from Stratagene (San Diego, CA). For coating of wells, 24- or 48-well culture dishes (Costar, Cambridge, MA) were incubated with 0.2 or 0.1 ml of 25 μg/ml FN or 120FN in PBS overnight (9–15 h) at 4°C. Wells were coated with solutions of ProNectinF (10 μg/ml in PBS) as described above. They were then washed three times with PBS and incubated in 0.2% BSA in PBS for 30 min at ambient temperature to reduce nonspecific binding to the tissue culture dish. The wells were then washed three times with PBS and used immediately. For coating with anti-FNR antibody, wells were incubated with BIIG2 IgG (50 μg/ml) in PBS overnight (9–15 h) at 4°C and processed further as described above. Larger plates were coated with FN or 120FN at a concentration of 3 $\mu g/cm^2$ or with BIIG2 IgG at a concentration of 1.5 $\mu g/cm^2$.

Biosynthetic Labeling of Proteins Secreted by RSF

RSF were added to ECM-coated wells and cultured in DME- LH for up to 48 h, after which the conditioned medium (CM) was removed and saved for later analysis. Cultures of RSF were biosynthetically labeled by incubation with 50–70 μCi/ml [^{35}S]methionine (Express Label, New England Nuclear, Boston, MA) for 3–4 h in methionine-free DME (GIBCO BRL, Gaithersburg, MD). Radiolabeled secreted proteins were precipitated from the CM with quinine sulfate and SDS or by immunoprecipitation with specific antibodies as described previously (Werb et al., 1989). Precipitates were analyzed by SDS-PAGE and fluorography (En^{3}Hance; New England Nuclear). Radiolabeled collagenase secreted into the culture supernatants was quantified by scanning the autoradiograms with a densitometer and software for image analysis, either an LKB-Pharmacia densitometer and GSXL software (Pharmacia LKB Biotechnology, Piscataway, NJ) or a Personal Densitometer with Image-Quant software (Molecular Dynamics, Sunnyvale, CA).

Analysis of Specific Proteinases

Proteinases in the CM harvested from cell cultures were analyzed by using substrate gel zymography as described previously (Werb et al., 1989). Proteins in the CM were separated on nonreducing 10% SDS-polyacrylamide gels that contained either 0.1% gelatin, or 0.1% casein. After electrophoresis, gels were soaked in 2.5% Triton X-100 to remove SDS and incubated for 18 h in substrate buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$). The gels were stained with Coomassie blue R250 (BioRad Labs., Richmond, CA). Zones containing proteinases appeared as clear bands on a blue background, but to allow better resolution they were printed as reverse images. The relative expression of gelatin- or casein-degrading proteinases in samples of CM harvested from cultures plated on FN or 120FN and incubated with antisense or sense c-*fos* oligonucleotides was compared by using the cleared areas of proteinase activity on the zymograms to estimate the level of expression of a particular proteinase. The data were compared by scanning densitometry and Image-Quant software (Molecular Dynamics).

For quantification of collagenase protein by immunoblotting, we applied several dilutions of CM directly to nitrocellulose by using a slot-blot filtration apparatus (Schleicher and Schuell, Keene, NH). Nonspecific sites on the membranes were blocked with a solution of 3% BSA in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), after which the membranes

were incubated with anti-collagenase or anti-stromelysin mAb in TBS containing 0.5% Tween-20 (Sigma) for 1-2 h at ambient temperature. Before and after incubation with horseradish peroxidase-conjugated anti-mouse IgG (Sigma), the membranes were washed three times for 20 min with 0.5% Tween-20 in TBS. Specific bands were detected by enhanced chemiluminescence (ECL) (Amersham) as described by the manufacturer. The films were scanned by using a densitometer and GelScan-XL software (Pharmacia LKB).

RNA Isolation and Analysis

Total cellular RNA was isolated from cultured RSF by the method of Chirgwin (1979). Alternatively, polyA$^+$ RNA was isolated by using a micro-Fast Track kit (Invitrogen, San Deigo, CA). RNA was separated on agarose gels, transferred to nylon membranes, and probed with ^{32}P-labeled DNA probes as described previously (Sambrook et al., 1989). Plasmids containing cDNA coding for rabbit interstitial collagenase (Frisch and Werb, 1989), human *c-fos* (kindly provided by Leslie Rall, San Francisco, CA), or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort et al., 1985) were used to generate probes.

Analysis of c-fos and c-jun

c-fos mRNA was analyzed by using the nucleic acid hybridization techniques described above. The c-Fos and c-Jun protein in nuclei harvested from cultures plated on ECM ligands was also analyzed by immunoblotting. Nuclear extracts were prepared by the method of Bos et al. (1988). The total protein content of these extracts was measured with the micro-BCA assay (Pierce, Rockford, IL), and samples normalized for protein content were reduced and separated on a 10% SDS-polyacrylamide gel as described by Harlow and Lane (1989). After electrophoresis, the proteins were transferred to Immobilon P membranes (Millipore, Boston, MA) (Harlow and Lane, 1989). Incubations with anti-c-Fos or anti-c-Jun antibodies were performed as described by the manufacturer, followed by incubation with secondary antibodies and detection with enhanced chemiluminescence as described above.

Immunofluorescence

In experiments that measured the expression of immunoreactive collagenase, cells were plated on acid-washed glass coverslips coated with ECM proteins as described above. At the indicated times after plating, coverslips were rinsed in PBS, and cells were fixed for 5 min in 2% paraformaldehyde in PBS. After fixation, cultures were made permeable by incubation in 0.25% Triton X-100 in PBS at ambient temperature for 2 min. Coverslips were rinsed in PBS, and nonspecific sites were blocked by incubation for several hours with a solution of 1% BSA in PBS before a 1-h incubation with a cocktail of five mAbs against rabbit collagenase (Werb et al., 1989). Cells were washed, incubated for 1 h with biotinylated goat anti-mouse IgG, rinsed, and incubated for 1 h with Texas red-streptavidin. The cells were photographed on a Zeiss photomicroscope II with phase contrast and epifluorescence, with the use of a 25× or 63× water immersion lens.

The coverslips that were stained with anti-Fos antibodies were fixed with ice-cold 4% paraformaldehyde in PBS and incubated on ice for 30 min, then made permeable at ambient temperature with 0.25% Triton X-100 in PBS for 5 min. Nonspecific sites were blocked by incubating coverslip cultures for at least 3 h with 0.2% BSA in PBS. Cells were then incubated with the anti-Fos antibody for 1 h at ambient temperature, followed by incubation for 10 h at 4°C. Coverslips were washed and then incubated with biotinylated anti-rabbit IgG, washed again with PBS, and incubated with Texas red-streptavidin. As a control for specificity, cells were incubated with a mixture of anti-Fos antibody and an excess of the peptide immunogen as described by the manufacturer. The cells were photographed as described above.

Reporter Plasmids

Reporter plasmids containing fragments of the human collagenase promoter inserted upstream from the chloramphenicol acyl transferase (CAT) gene were generously provided by Peter Herrlich and Hans Rahmsdorf, Karlsruhe, Germany. These plasmids, −1200 tkCAT, −139 tkCAT, and −66 tkCAT (see Fig. 1), contained the indicated fragments of the human

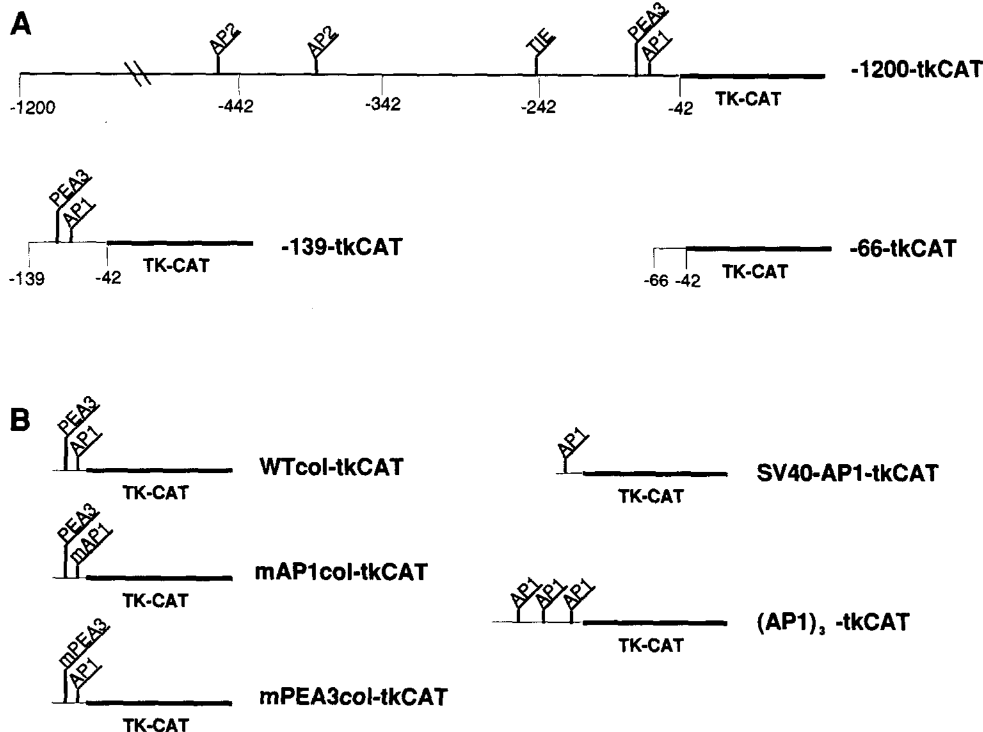


Figure 1. Reporter plasmids used in this study. (*A*) The collagenase-reporter constructs −1200 tkCAT, −139 tkCAT, and −66 tkCAT contain segments of the human collagenase promoter (from −1200/−42, −139/−42, and −66/−42 bp) inserted upstream from the minimal thymidine kinase promoter and the gene coding for CAT in pBLCAT2 (Angel et al., 1987*a*). The inducible elements include an AP1 site that is also known as a TPA-responsive element at −72/−67, a PEA3 site at −90 to −82, a TGF-β inhibitory element (TIE) at −246 to −237, and AP2 sites at −484/−464 and −234/−214 (Angel et al., 1987*a,b*; Gutman and Wasylyk, 1990; Kerr et al., 1990). (*B*) Promoter constructs containing only the AP1 and PEA3 sequences from the human collagenase promoter, or derivatives of these sequences, were made as described in Materials and Methods. The construct WTcol-tkCAT contains the native AP1 and PEA3 sequences from the human collagenase gene (−90/−67) inserted into pBLCAT2. In mAPcol-tkCAT, the AP1 site was disrupted by changing AT to TG, which prevents formation of complexes by this oligonucleotide and AP1 in gel shift assays (Gutman and Wasylyk, 1990). A G to A substitution in the PEA3 sequence generated the mPEAcol-tkCAT construct; oligonucleotides with this sequence do not form complexes with Ets1 in gel shift assays (Gutman and Wasylyk, 1990). (*C*) Reporter constructs containing AP1 sequences from other genes were also tested. The (AP1)$_3$ plasmid contains three tandem copies of the AP1 sequence from the proliferin gene. The plasmid SV40AP1 contains one copy of the SV40 TPA-responsive element inserted into pUC19tkCAT (Moye-Rowley et al., 1989).

collagenase promoter inserted in front of a minimal thymidine kinase promoter in the plasmid pBLCAT2, first described by Angel et al. (1987*a*). A CAT reporter plasmid, SV40-AP1-tkCAT, containing only the minimal SV40 AP1 site (Harshman et al., 1988), was generously provided by W. S. Moye-Rowley (University of Iowa, Iowa City, IA). The CAT reporter construct $(AP1)_3$-tkCAT, which contains three tandem repeats of the AP1 site in the proliferin promoter (Diamond et al., 1990), was provided by Susan Logan (University of California, San Francisco). The expression of β-galactosidase from cotransfected ΔGRE-βgal construct, which contains a Rous sarcoma virus promoter, was used to normalize for transfection efficiency. The plasmids pCH110 (Pharmacia) and ΔGRE-βgal, provided by J. A. Miner (University of California, San Francisco), were used to normalize for transfection efficiency.

The plasmids mAPcol-tkCAT, mPEAcol-tkCAT, and WTcol-tkCAT were constructed with synthetic oligonucleotides representing the AP1 and PEA3 sites in the human collagenase promoter (Angel et al., 1987*a*). WTcol-tkCAT (AGC TTG AGG ATG TTA TAA AGC ATG AGT CAG) contains the AP1 and PEA3 sites present in the human collagenase promoter. mAPcol-tkCAT (AGC TTG AGG ATG TTA TAA AGC tgG AGT CA) contains a substitution of AT to TG in the AP1 site; in gel shift assays, oligonucleotides containing this substitution do not interact with AP1 (Gutman and Wasylyk, 1990). mPEAcol-tkCAT (AGC TTG AaG ATG TTA TAA AGC ATG AGT CA) contains a substitution of G to A in the PEA3 site; in gel shift assays, oligonucleotides containing this substitution do not interact with PEA3 (Gutman and Wasylyk, 1990). The annealed oligonucleotides were subcloned into the BamHI–HindIII site in pBLCAT2. The insertion of the oligonucleotides was confirmed by hybridization analysis with end-labeled oligonucleotides.

Synthesis of Oligonucleotides

Oligonucleotides were synthesized on a PCR-Mate, model No. 391, from Applied Biosystems (Foster City, CA), with columns and reagents from Applied Biosystems. Full-length oligonucleotides were purified with oligonucleotide purification cartridges as supplied and described by Applied Biosystems. Antisense and sense *c-fos* oligonucleotides represented the 18 residues from and including the start codon for *c-fos* (Verma et al., 1986): ATG ATG TTC TCG GGC TTC AA (sense *c-fos*, FOS-OLIGO); TTG AAG CCC GAG AAC ATC AT (antisense *c-fos*, SOF- OLIGO).

Transfections

Except for experiments in which antisense and sense oligonucleotides were added to cells concurrently with the reporter construct, CAT plasmids were introduced into cells either by calcium phosphate coprecipitation of DNA (Sambrook et al., 1989) or by the Lipofectin reagent (GIBCO BRL). Typically, 10–20 μg of CAT construct and 2 μg of β-galactosidase construct were coprecipitated with calcium phosphate (Sambrook et al., 1989); the precipitates were added to a 75-cm^2 flask of cells (subcultured 24 h before transfection) in DME containing 10% FBS. The cultures were incubated with the DNA precipitate for 5–6 h and allowed to recover overnight (9–15 h) in DME-LH, after which the cells were plated on ECM-coated wells (Costar 6-well clusters) as described for individual experiments. In transfection experiments with the Lipofectin reagent, a 100-μl aliquot containing 10 μg of CAT construct and 2 μg of β-galactosidase construct was mixed with 100 μl of a 50% solution of Lipofectin in DME. The DNA encapsulated in Lipofectin was added to a 75-cm^2 flask of cells (subcultured 24 h before transfection) in unsupplemented DME, and the cultures were incubated in this mixture overnight (9–15 h). These cells were then subcultured and plated on ECM-coated wells as described for individual experiments. Cell extracts were prepared 50 h after plating on ECM-coated wells.

In some experiments both oligonucleotides and plasmids were introduced into cells by electroporation. In these experiments, RSF in suspension were washed three times with DME supplemented with 20 mM Hepes, pH 7.5, and resuspended in Hepes-buffered DME at a concentration of 6.2×10^7 cells per ml. Aliquots (0.8 ml) of this cell suspension were added to disposable cuvettes (GIBCO BRL), along with 60 μg of –139 tkCAT and either sense *c-fos* (FOS-OLIGO) or antisense *c-fos* (SOF-OLIGO) oligonucleotides (25 μM final concentration). The cuvettes were placed on ice for 2 min, and cells were electroporated in a water-filled electroporation chamber (Cellporator, GIBCO BRL) with settings of 280 V and 500 μF. Cells were allowed to recover for 3 min at ambient temperature before removal from the cuvette and plating on ECM-coated wells in DME-LH supplemented with the appropriate oligonucleotide. Cell extracts were prepared after 50 h of incubation.

CAT Assays

Transcriptional response as determined by expression of CAT enzyme activity in cell extracts transfected with CAT plasmids was measured in assays that used either ^{3}H-labeled chloramphenicol and thin layer chromatography to separate the acetylated chloramphenicol from unacetylated chloramphenicol (Gorman et al., 1982; Sambrook et al., 1989), or ^{14}C-labeled coenzyme A and ethyl acetate to extract acetylated chloramphenicol products (Sleigh, 1986; Sambrook et al., 1989). Assay of β-galactosidase activity was performed as described by Sambrook et al. (1989).

Results

Elements in the −139/−42 Segment of the Human Collagenase Promoter Regulate Transcription in Cells Adhering to Fibronectin Fragments or to the Antibody to the $\alpha_5\beta_1$ Integrin Fibronectin Receptor

To define the elements in the collagenase promoter that are required for the regulation of collagenase by integrins or adhesion-derived signals, we first established that the collagenase-CAT promoter-reporter plasmids transiently introduced into cells were regulated coordinately with the endogenous collagenase gene. We used constructs in which segments of the human collagenase gene between positions −1200 and −42 bp, −139 and −42 bp, or −66 and −42 bp (relative to the transcription start site) were placed upstream of the minimal thymidine kinase promoter and the bacterial gene for CAT in pBLCAT2 (Angel et al., 1987*a*; Fig. 1). We used reporter constructs that contain hybrid promoters because the regulation of these constructs in many types of cells by growth factors, cytokines, and drugs has been characterized (Angel et al., 1987*a*,*b*; Kim et al., 1990; Lafyatis et al., 1990). The transfected cells were then plated in DME-LH in wells that were coated with FN, or the 120-kD chymotrypsin fragment of FN (120FN), or BIIG2, an adhesion-perturbing mAb that recognizes an epitope on the α_5 subunit of the $\alpha_5\beta_1$ FNR (anti-FNR antibody). Because the induction of both the endogenous collagenase gene and the promoter-reporter constructs driven by the collagenase promoter in response to phorbol esters has been well characterized (Frisch and Werb, 1989; Werb et al., 1986; Angel et al., 1987*a*,*b*; Auble and Brinckerhoff, 1991), we included cells plated on FN and treated with 12-O-tetradecanoyl phorbol-13-acetate (TPA) as a positive control in all experiments.

The expression of −1200 tkCAT and of −139 tkCAT was induced similarly in cells plated on 120FN or on anti-FNR antibody, relative to cells plated on FN (Fig. 2). In contrast, in cells transfected with −66 tkCAT, minimal expression was observed under all plating conditions. These data indicate that the same ECM ligands that lead to induction of the endogenous collagenase gene (120FN and anti-FNR antibody, but not intact FN; Werb et al., 1989) also lead to increased transcription of the promoter-reporter constructs, and that the elements necessary for the induction of collagenase in these cells are included in the –139/–67 segment of the promoter. However, –1200 tkCAT and −139 tkCAT did not behave identically under all conditions, because the induction of –1200 tkCAT was higher than that for the –139 tkCAT construct in cells plated on FN/TPA. This indicates that there are additional sequences upstream of –139 that respond positively to TPA.

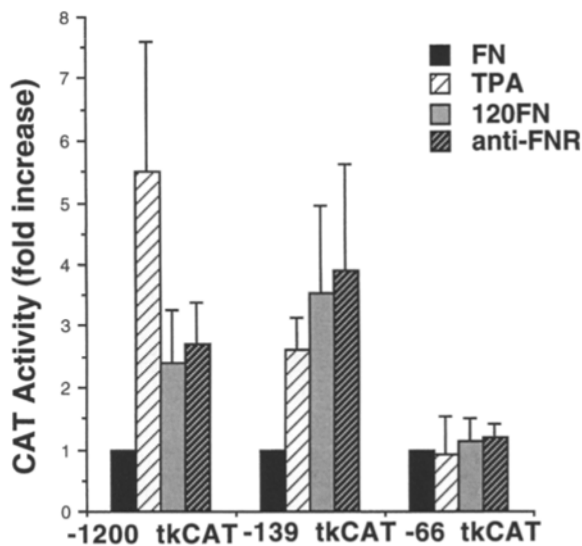


Figure 2. The expression of promoter-reporter constructs containing sequences from the human collagenase promoter is induced in RSF plated on 120FN or anti-FNR antibody. RSF were transfected with 20 μg of the indicated collagenase-CAT construct and 2 μg of ΔGRE-βgal, a constitutively expressed vector for β-galactosidase, by incubation with calcium phosphate-DNA precipitates for 6 h. The precipitates were removed and cells were allowed to recover overnight (9–14 h) in DME-LH. The transfected RSF were subcultured and plated in DME-LH on ECM-coated wells. Cell extracts were prepared from RSF that were cultured for 48–50 h, and the expression of CAT was determined from enzyme activities. CAT activities were normalized to β-galactosidase activity and are presented as the ratio of the CAT activity in cell extracts from RSF transfected with a collagense-tkCAT construct, then plated on 120FN, anti-FNR antibody (BIIG2), or FN/TPA, to the CAT activity in cell extracts prepared from RSF transfected with the same construct and then plated on FN. The data shown are the average of results from nine experiments, in which six different isolates of RSF were used. The induction of −1200 tkCAT and −139 tkCAT constructs in cells plated on 120FN or anti-FNR antibody was upregulated to about the same extent relative to that in cells plated on FN; however, the absolute expression of –139 tkCAT was higher than that of −1200 tkCAT. For example, in two sets of experiments with RSF of similar passage number that were isolated from the same rabbit, the CAT activity from −1200 tkCAT in RSF plated on FN was 3.3 ± 0.1 times that of the parent pBLCAT2 vector, the CAT activity from −139 tkCAT in RSF plated on FN was 15 ± 1.2 times that of the parent pBLCAT2 vector, and the CAT activity from −66 tkCAT was 1.0 ± 0.2 times that of the parent pBLCAT2. In these experiments the baseline CAT activity from pBLCAT2 was 500 ± 50 cpm.

Both the AP1 and PEA3 Sites Are Required to Activate Collagenase Transcription in Cells Adhering to Anti-FNR Antibody or Fibronectin Fragments

Two elements in the −139/−67 region of the collagenase promoter—an activator protein-1 (AP1) site at −72/−67 and a polyomavirus enhancer activator-3 (PEA3) site at −90/−82—have been shown to confer inducibility by growth factors and TPA (Angel and Karin, 1992; Gutman and Wasylyk, 1990 and the references therein). Accordingly, we determined the expression of a −90/−67 promoter-reporter construct (WTcol-tkCAT), which contains only the PEA3 and AP1 sequences from the human collagenase promoter, in cells plated on FN, 120FN, or anti-FNR antibody. Expression of WTcol-tkCAT was upregulated in cells plated on 120FN or anti-FNR antibody (Fig. 3). Although the overall amount of CAT activity in cells transfected with WTcol-tkCAT was lower than that from cells transfected with −139 tkCAT (Fig. 2), the expression of these constructs in cells plated on inductive ligands was induced to a similar extent, relative to that in cells plated on FN. Thus, the AP1 and PEA3 sites are important for the regulation of collagenase in response to signaling cascades initiated by integrins. In contrast, the expression of a reporter construct with only one AP1 site was not induced in RSF plated on 120FN or anti-FNR antibody (data not shown). However, expression of $(AP1)_3$-tkCAT, a construct containing three tandem repeats of the AP1 site from the proliferin gene, was induced to a similar extent as that of WTcol-tkCAT and −139 tkCAT in cells plated on 120FN or anti-FNR antibody.

To assess cooperation between AP1 and PEA3 sites in the collagenase promoter, we compared the regulation of the minimal promoter, WTcol-tkCAT, with derivatives that contain substitutions in either the AP1 site (mAPcol-tkCAT) or the PEA3 site (mPEAcol-tkCAT). Mutation of either the AP1 or the PEA3 site in WTcol-tkCAT ablated transcription from this minimal promoter in cells plated on all substrates (Fig. 3). These data suggest that both AP1 and PEA3 sites are required for integrin- and ECM-dependent regulation of the collagenase gene.

The Accumulation of Nuclear c-Fos and c-Jun Precedes the Induction of Collagenase in Cells Plated on 120FN

Proteins of the Jun family regulate transcription from AP1 sites, binding to AP1 sites with high affinity as heterodimeric complexes of Fos and Jun family members or with lower affinity as homodimers of Jun family members (for review see Angel and Karin, 1992; Brenner et al., 1989). In response to other stimuli, increases in the expression of collagenase are often preceded by transient increases in the expression of c-*fos* and/or c-*jun* mRNA, implying that the increase in transcription of collagenase is mediated by heterodimeric complexes of Fos and Jun. Because the AP1 site in the minimal collagenase promoter, WTcol-tkCAT, is essential for the upregulation of collagenase in response to integrin-derived signals, we next compared the accumulation of c-*fos* mRNA and protein in cells that were plated on inductive or noninductive ligands for the FNR. We examined c-*fos* and GAPDH mRNA prepared from cells that were plated for 1 h on FN, 120FN, anti-FNR antibody, or a poly-RGD compound (ProNectinF) by RNA blot analysis, using ^{32}P-labeled cDNA probes for c-*fos* and GAPDH. Expression of c-*fos* mRNA increased in cells plated on 120FN, anti-FNR antibody, or FN/TPA, relative to that in cells plated on FN (Fig. 4 *A*). An increase in the expression of c-*fos* mRNA at 1 h correlated with the upregulation of collagenase mRNA at 24 h (Fig. 4 *B*).

We then used immunocytochemistry with an antibody generated against a c-Fos peptide to detect c-Fos protein in cells (Fig. 5). RSF cultured on FN substrates in DME-LH had very low amounts of nuclear c-Fos. In contrast, RSF plated on 120FN for 1 h exhibited a striking increase in

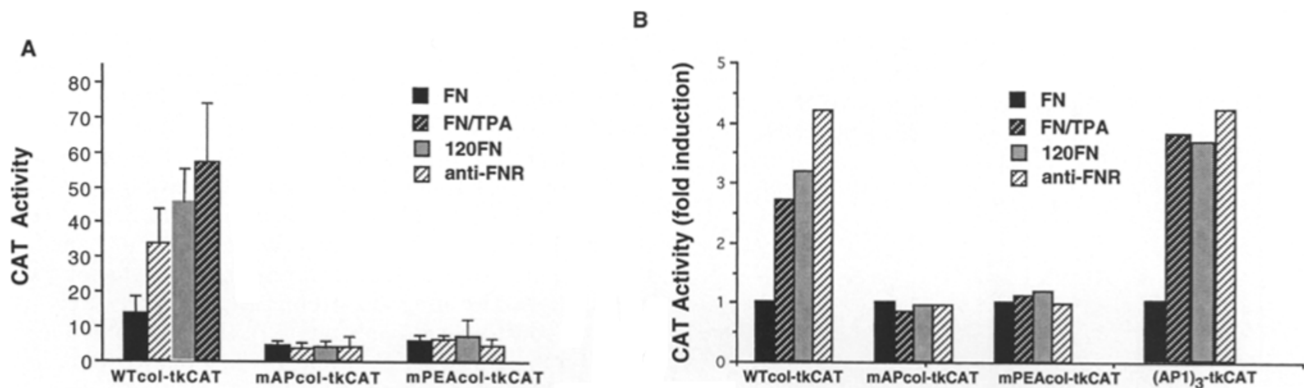


Figure 3. The AP1 and PEA3 sites are involved in the regulation of collagenase by ECM ligands or TPA. RSF were transfected with 20 μg of the indicated CAT reporter plasmid containing segments of the human collagenase promoter, along with 2 μg of ΔGRE-βgal, by incubation with calcium phosphate-DNA precipitates as described in the legend to Fig. 2. After transfection, RSF were subcultured and plated in DME-LH on ECM-coated wells; one set of cultures plated on FN were cultured in DME-LH supplemented with 100 ng/ml TPA. Cell extracts were harvested 48–50 h after plating on ECM substrates, and the expression of CAT was measured by comparing CAT enzyme activity in cell extracts. (*A*) CAT activity was normalized to β-galactosidase activity, and the data are expressed as the ratio of CAT activity from collagenase promoter-reporter plasmids to that seen in cells transfected with the parent pBLCAT2 construct. The data are presented as mean ± SD of the results of five experiments. In cells transfected with WTcol-tkCAT, CAT expression in cells plated on 120FN, anti-FNR antibody, or FN/TPA was significantly different ($p < 0.01$, < 0.005, < 0.005, respectively; Student's t test) from that seen in cells plated on FN. CAT activity in extracts prepared from cells transfected with pBLCAT2 ranged from 200 to 500 cpm in the individual experiments. (*B*) The data from *A*, presented as the ratio of CAT activity in cell extracts from RSF transfected with a minimal promoter construct and plated on agonist ECM ligands, to the CAT activity in cell extracts prepared from RSF transfected with the same construct and plated on FN.

staining for nuclear c-Fos. In control experiments, staining of nuclear c-Fos was increased in RSF treated with TPA for 1 h, and no staining was detected in TPA-treated cells stained with anti-Fos antibody preparations that had been preincubated with the peptide immunogen (Fig. 5). The nuclear accumulation of c-Fos at 1 h correlated well with the expression of collagenase in cells at 24 h. To quantify the response, we scored cells for immunoreactive c-Fos or collagenase (five randomly selected fields, 25–50 cells per field per treatment, were counted in two experiments). In RSF plated on FN, 16% stained weakly for nuclear c-Fos, whereas immunoreactive c-Fos staining was detected in 80% of cells plated on 120FN and in 89% of cells plated on FN/TPA. The number of cells staining for c-Fos closely paralleled the number of cells with immunoreactive, cell-associated collagenase. Few of the cells plated on FN contained immunoreactive collagenase (10%), whereas 76% of cells plated on 120FN and 80% of cells plated on FN/TPA were positive for collagenase.

To characterize the timing of this increase in c-Fos, we immunoblotted nuclear extracts of RSF adhering to either FN or 120FN for 10, 20, 40, or 60 min with anti–c-Fos antibodies (Fig. 6 *A*). In RSF plated on 120FN nuclear c-Fos increased as early as 10 min after adhering to 120FN, and continued to increase over the next hour, relative to cells plated on FN. c-Fos was also analyzed in immunoblots of nuclear extracts prepared from quiescent, adherent RSF that were treated with 100 ng/ml TPA, or from quiescent, adherent RSF cultures (Fig. 6 *B*). As expected, we saw an increase in nuclear Fos in RSF soon after the addition of TPA. For a biochemical analysis of both proteins of the AP1 complex, we immunoblotted nuclear extracts prepared from cells adhering to ECM substrates for 1 h with an anti-Fos antibody and an anti-peptide Jun antibody (Fig. 6 *C*). Nuclear c-Fos and c-Jun were substantially higher in nuclei harvested from cells plated on 120FN than in nuclei harvested from cells plated on FN (Fig. 6 *C*). Nuclear Fos and Jun were also higher in cells that were plated

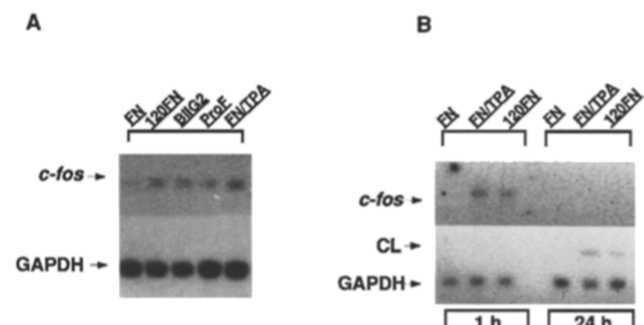


Figure 4. mRNA coding for c-*fos* increases in RSF plated on 120FN, anti-FNR antibody, or FN/TPA. (*A*) RSF were plated on wells coated with FN, 120FN, anti-FNR antibody (BIIG2), or the poly-RGD compound ProNectinF (*ProF*), and cultured in DME-LH; as a positive control, one sample of RSF plated on FN was treated with 100 ng/ml TPA (*FN/TPA*). After incubation for 1 h, polyA$^+$ RNA was isolated from cell lysates. Equal amounts of polyA$^+$ RNA were separated on agarose gels, and the RNA was transferred to nylon membranes and probed with ^{32}P-labeled cDNA inserts coding for c-*fos* and GAPDH. (*B*) The increase in c-*fos* mRNA in RSF plated on 120FN or FN/TPA correlates with the expression of collagenase. RSF were plated in DME-LH on wells coated with FN or 120FN or plated on FN and treated with 100 ng/ml TPA (*FN/TPA*). After 1 h or 24 h of incubation, total RNA was isolated from the cells. 20-μg samples of RNA were separated by electrophoresis, and RNA was transferred to nylon membranes, which were hybridized with ^{32}P-labeled cDNA inserts coding for c-*fos*, collagenase (*CL*), and GAPDH.

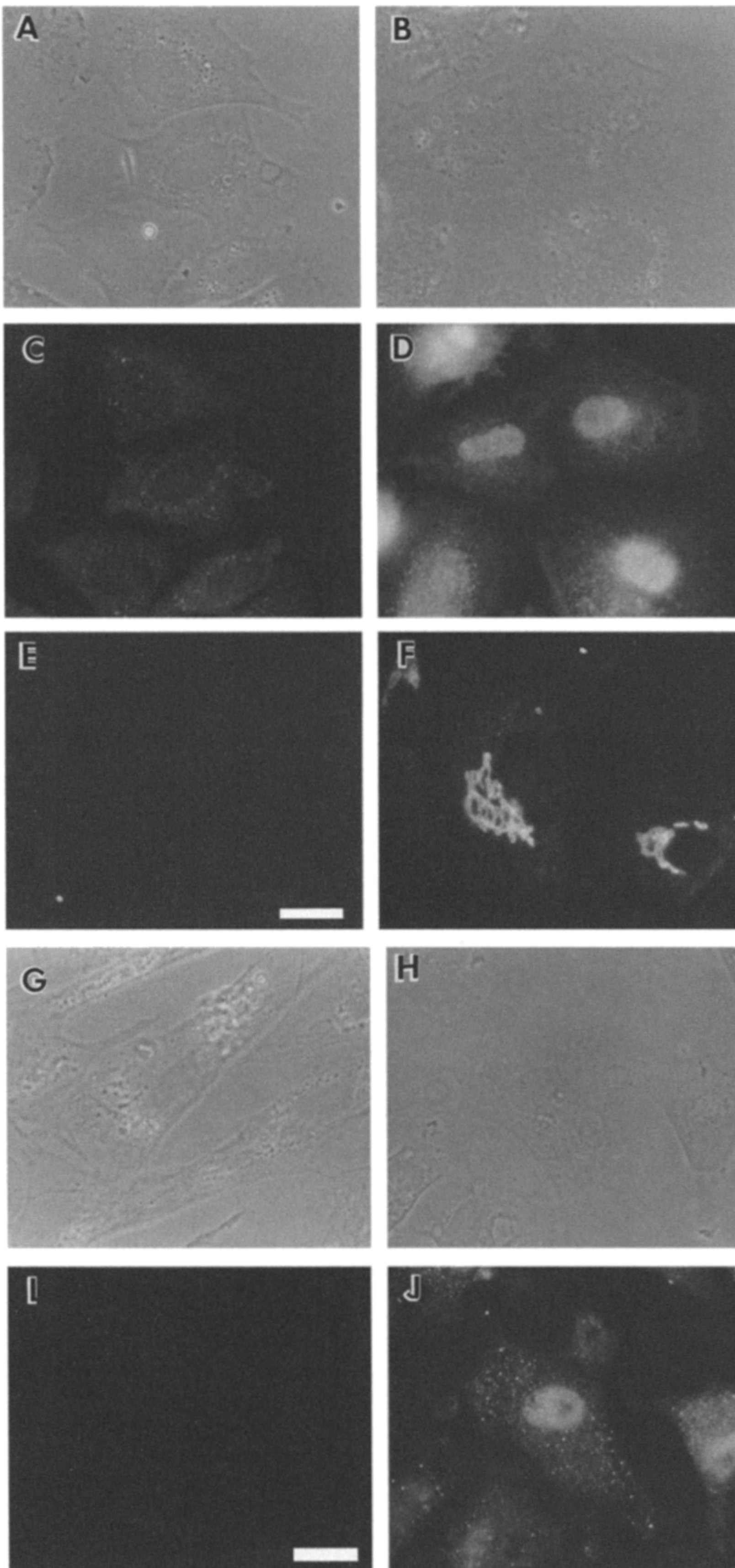


Figure 5. Analysis of nuclear c-Fos and c-Jun. We used a polyclonal antibody generated against a Fos peptide sequence (CRB Biologics) to analyze the immunoreactive c-Fos in RSF plated on FN or 120FN. RSF were plated in DME-LH on coverslips that were coated with FN (*A*, *C*, and *E*) or 120FN (*B*, *D*, and *F*) and incubated for 1 h (*A–D*) or 24 h (*E* and *F*). The cultures were terminated by addition of paraformaldehyde and processed for immunocytochemistry to analyze the expression of Fos (*A–D*) or cell-associated collagenase (*E* and *F*). As a positive control for the analysis of Fos protein by immunocytochemistry, RSF were cultured in DME containing serum on glass coverslips for 5 d, treated with 100 ng/ml TPA in DME-LH for 1 h, fixed, and processed for analysis of Fos protein (*G–J*). In one set of cultures, the anti-Fos peptide antibody was preincubated with an excess of the peptide immunogen (*G* and *I*). Bar equals 20 μm.

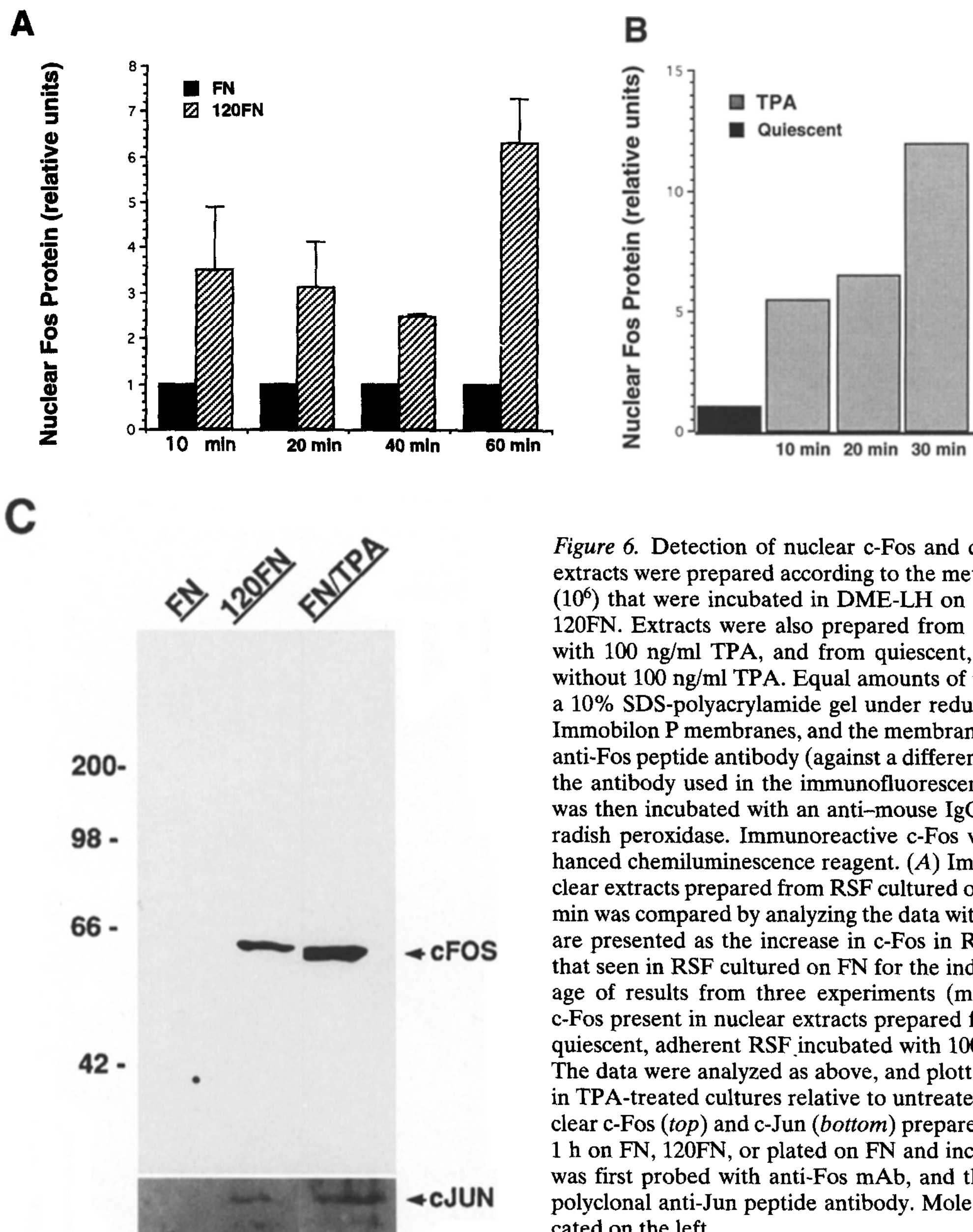


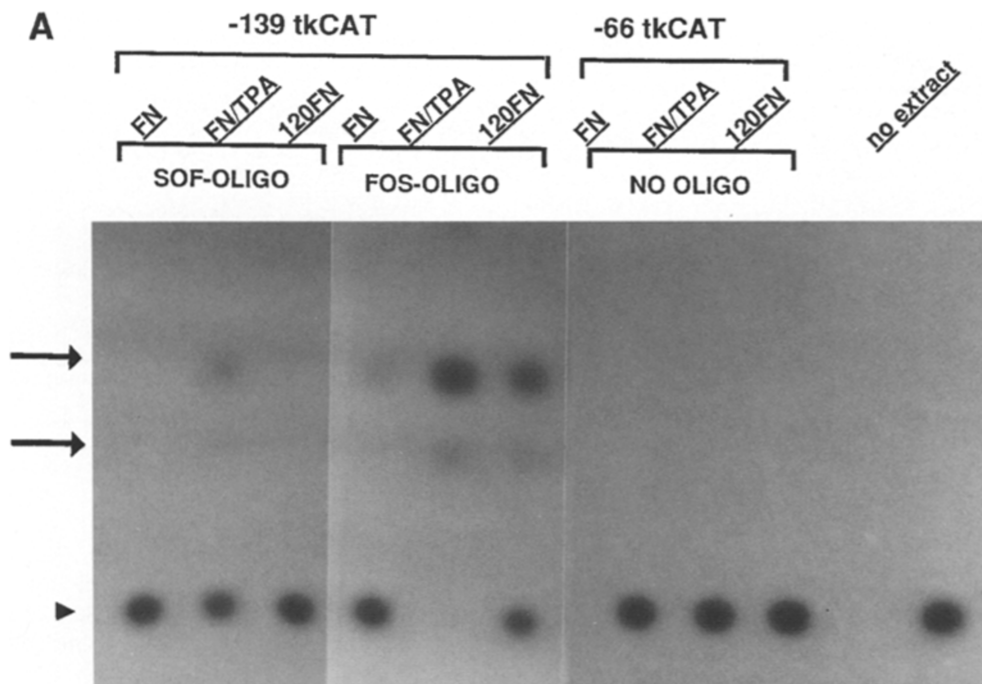
Figure 6. Detection of nuclear c-Fos and c-Jun by immunoblotting. Nuclear extracts were prepared according to the method of Bos et al. (1988) from RSF (10^6) that were incubated in DME-LH on 100-mm dishes coated with FN or 120FN. Extracts were also prepared from RSF plated on FN and incubated with 100 ng/ml TPA, and from quiescent, adherent cells incubated with or without 100 ng/ml TPA. Equal amounts of nuclear protein were separated on a 10% SDS-polyacrylamide gel under reducing conditions and transferred to Immobilon P membranes, and the membranes were probed with a monoclonal anti-Fos peptide antibody (against a different domain of the c-Fos protein than the antibody used in the immunofluorescent studies (Fig. 5). The membrane was then incubated with an anti-mouse IgG antibody conjugated with horseradish peroxidase. Immunoreactive c-Fos was detected by means of the enhanced chemiluminescence reagent. (*A*) Immunoreactive c-Fos present in nuclear extracts prepared from RSF cultured on FN or 120FN for 10, 20, 40, or 60 min was compared by analyzing the data with scanning densitometry. The data are presented as the increase in c-Fos in RSF cultured on 120FN relative to that seen in RSF cultured on FN for the indicated time. The data are an average of results from three experiments (mean ± SD). (*B*) Immunoreactive c-Fos present in nuclear extracts prepared from quiescent, adherent RSF and quiescent, adherent RSF incubated with 100 ng/ml TPA for 10, 20, or 30 min. The data were analyzed as above, and plotted as the increase in nuclear c-Fos in TPA-treated cultures relative to untreated cultures. (*C*) Immunoblot of nuclear c-Fos (*top*) and c-Jun (*bottom*) prepared from RSF that were cultured for 1 h on FN, 120FN, or plated on FN and incubated with TPA. The membrane was first probed with anti-Fos mAb, and then stripped and reprobed with a polyclonal anti-Jun peptide antibody. Molecular mass markers (kD) are indicated on the left.

on FN/TPA. Because the anti-Fos antibody was generated against a different domain of the c-Fos protein than was the antibody used in the immunofluorescent studies, these data confirm the nuclear localization of c-Fos in RSF plated on agonistic ligands for the FNR.

Addition of Antisense c-fos Oligonucleotides to Cells Diminishes the Expression of Collagenase in RSF Plated on 120FN

The experiments described thus far suggest that both Fos and Jun nuclear proteins may play a role in the increased transcription of collagenase induced by integrin-mediated signaling. If the increase in collagenase expression is dependent on the increased expression of c-*fos*, preventing this increase in c-*fos* expression should diminish the expression of collagenase. Such an approach has been used successfully to analyze the role of c-*fos* in regulating the expression of stromelysin by epidermal growth factor (Kerr et al., 1988; McDonnell et al., 1990) and by interleukin-1 (Quinones et al., 1989; Buttice et al., 1991; Buttice and Kurkinen, 1993). Accordingly, we used synthetic DNA oligonucleotides that were complementary to c-*fos* mRNA sequences (antisense c-*fos* oligonucleotides, SOF-OLIGO) or identical to c-*fos* mRNA sequences (sense c-*fos* oligonucleotides, FOS-OLIGO), introducing them into RSF by electroporation along with a collagenase promoter-CAT construct.

Transfection of RSF with a mixture of −139 tkCAT and SOF-OLIGO markedly decreased (to <6% of FOS-OLIGO) both the induced expression of −139 tkCAT in cells plated on 120FN and its basal expression in cells plated on FN (Fig. 7, *A*, *B*, and *C*). FOS-OLIGO did not significantly affect either the induced or basal expression of −139



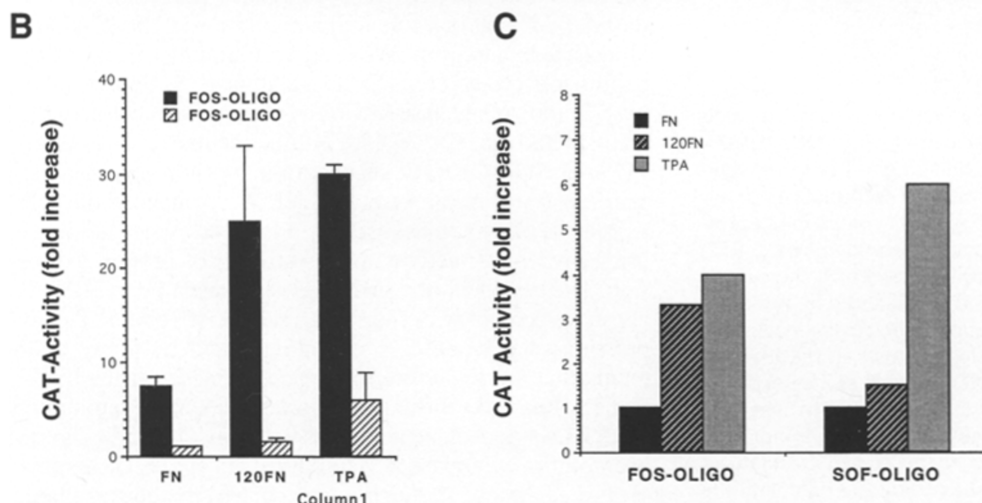


Figure 7. Antisense c-*fos* oligonucleotides compromise the induction of collagenase in cells plated on inductive ECM ligands. (*A*) Oligonucleotides that were complementary (antisense c-*fos* oligonucleotides, *SOF-OLIGO*) or identical (sense c-*fos* oligonucleotides, *FOS-OLIGO*) to c-*fos* mRNA sequences were introduced into RSF by electroporation along with −139 tkCAT. The electroporated cells were plated in DME-LH on wells coated with FN or 120FN, and cultures were incubated for 70 h. As a positive control, one set of cultures was incubated in DME-LH containing 100 ng/ml TPA (*FN/TPA*). RSF were cultured in DME-LH supplemented with the indicated oligonucleotide at a concentration of 25 μM; oligonucleotides were replenished every 12 h during the 70 h of culture. Cell extracts were prepared, and CAT expression was measured by comparing CAT enzyme activity from equal amounts of cell extracts. Acetylated chloramphenicol (*arrows*) was separated from chloramphenicol (*arrowhead*) by thin layer chromatography, and the chromatograms were analyzed by autoradiography. The construct −66 tkCAT, which is just 3′ to the AP1 site, was not upregulated by TPA or in cells plated on 120FN. (*B*) The samples shown in *A* were quantified by excising and counting the acetylated chloramphenicol and the unmodified chloramphenicol. The data were calculated as pmoles acetylated CAT per milligram of cell extract (mean of two experiments, each performed in duplicate; bar ± SD), and plotted with the level of CAT activity in RSF plated on FN and incubated with SOF-OLIGO set to 1. (*C*) The data from *B* were replotted to show the induction of −139 tkCAT in cultures incubated with FOS-OLIGO or with SOF-OLIGO; in each condition the CAT activity in RSF plated on FN was set to 1.

tkCAT. CAT activity in cells treated with FOS-OLIGO plated on FN or 120FN was 70–80% of that in cells incubated without oligonucleotides. CAT expression in cells transfected with the −66 tkCAT construct was close to that of the parent pBLCAT2 plasmid backbone (Fig. 7 *A*). The expression of −139 tkCAT in RSF incubated with SOF-OLIGO was, in all cases, lower than that measured in the baseline condition (RSF incubated with FOS-OLIGO that were plated on FN). Overall, the expression of collagenase was diminished when the expression of c-*fos* was compromised. However, in RSF cultured with SOF-OLIGO, the ratio of −139 tkCAT induced in cells plated on 120FN relative to that seen on FN was lower than that seen in control cultures, while incubation of cultures with SOF-OLIGO did not affect the fold-induction of this construct in cultures that were treated with TPA.

We then analyzed the expression of the endogenous collagenase gene in cells incubated with FOS-OLIGO or SOF-OLIGO. The pattern of radiolabeled proteins secreted by RSF incubated with FOS-OLIGO was the same as that of RSF cultured without oligonucleotides; in both cases the expression of collagenase increased in RSF plated on 120FN (Fig. 8). The addition of SOF-OLIGO to cultures caused a general and specific effect on the pattern of radiolabeled proteins that were secreted by RSF cultured on FN or 120FN (Table I). We compared the effect of SOF-OLIGO on the expression of p205 (a protein species with the apparent molecular mass of 205 kD, the syn-

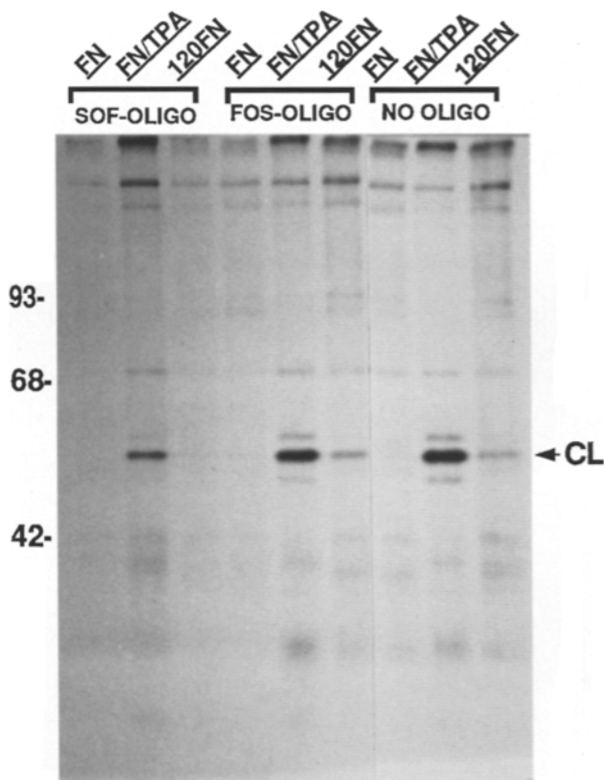


Figure 8. The regulation of endogenous collagenase in cells plated on 120FN is diminished by incubation with SOF-OLIGO. Cells transfected with −139 tkCAT and FOS-OLIGO or SOF-OLIGOs were plated in DME-LH on a 48-well plate that was coated with FN or 120FN. Some RSF were incubated in the presence of 100 ng/ml TPA with or without oligonucleotides (25 μM) (*FN/TPA*). RSF were cultured for 70 h, after which the culture supernatants were removed and saved for analysis by zymography. The cultures were biosynthetically labeled by incubation with [^{35}S]methionine. The radiolabeled proteins in the medium were concentrated and analyzed by SDS-PAGE and autoradiography. The migration of collagenase (*CL*), identified by immunoprecipitation (Werb et al., 1989; Tremble et al., 1993), is indicated on the right. The protein species, p205, that was used as an indicator of baseline metabolism is also indicated on the right, and the migration of the molecular mass standards (kD) is indicated on the left.

thesis of which is similar in RSF cultured on FN and 120FN) with the effect that SOF-OLIGO had on the expression of collagenase (Fig. 8, Table I). In control cultures, incubated with FOS-OLIGO, the expression of secreted p205 increased twofold in cultures plated on 120FN, relative to FN, whereas the expression of collagenase increased fourfold. In cultures incubated with SOF-OLIGO the expression of p205 and collagenase in RSF plated on FN did not differ from that seen for cultures plated on 120FN and incubated with SOF-OLIGO. Comparison of the ratio of values representing p205 or collagenase protein incubated with SOF- and FOS-OLIGO (SOF/FOS ratio) shows that apart from an effect on the baseline metabolism, the expression of collagenase is further affected by incubation with antisense SOF-OLIGO.

We have previously noted that, like collagenase, the expression of both stromelysin-1 and the 92-kD gelatinase B is upregulated in cells plated on 120FN or anti-FNR antibody relative to that in cells plated on FN (Werb et al., 1989; Tremble et al., 1994). The promoters of stromelysin 1, gelatinase B, and collagenase contain AP1 sites that are known to participate in the regulation of these genes by growth factors and phorbol esters (Sato and Seiki, 1993; Buttice and Kurkinen, 1993; Huhtala et al., 1991); we wondered if c-*fos* plays a role in the regulation of the 92-kD gelatinase B by integrins. We used zymography to analyze the expression of secreted 72-kD gelatinase A, 92-kD gelatinase B and collagenase in CM harvested from cultures that were plated on FN or 120FN and incubated with either FOS- or SOF-OLIGO, incorporating either gelatin or casein substrates in the resolving gel. We compared the relative levels of proteinases secreted by cells by densitometry, using proteolytic activity as an estimate of protein expression. The results of one such analysis, presented in Table II, are in agreement with the results shown in Table I. The expression of gelatinase A is not regulated by many treatments that are known to induce collagenase, stromelysin-1 and gelatinase B (Birkedal-Hansen, 1993), and examination of DNA sequences suggests that there are differences in the promoter of gelatinase A (Huhtala et al., 1991). The expression of gelatinase A was not upregulated in cultures plated on 120FN (Werb et al., 1989; Table II); however, there was a decrease in the overall level of gelatinase A in cultures incubated with SOF-OLIGO, probably resulting from an effect on baseline protein synthesis (Table II). The increase in expression of gelatinase B and collagenase in cultures plated on 120FN was not affected in cultures incubated with FOS-OLIGO (Table II). In contrast, the expression of gelatinase B and collagenase was significantly decreased in cultures incubated with SOF-OLIGO; this decrease was in addition to the effect of SOF-OLIGO on baseline protein synthesis (Table II; compare SOF/FOS ratio of gelatinase A with the ratio for gelatinase B or collagenase).

Table I. The Regulation of Radiolabeled, Secreted Collagenase Protein in Cultures Incubated with SOF-OLIGO

	FN		120FN	
	p205	Collagenase	p205	Collagenase
	units	*units*	*units*	*units*
FOS-OLIGO	50 ± 5	16.0 ± 5.7	100 ± 27	62.0 ± 20.0
SOF-OLIGO	30 ± 9	5.0 ± 1.0	30 ± 9	5.5 ± 2.4
Ratio (SOF/FOS)	0.6	0.3	0.3	0.09

RSF were plated on FN or 120FN and incubated with SOF-OLIGO or FOS-OLIGO, exactly as described in the legend to Fig. 8. The cultures were biosynthetically labeled by incubation with [^{35}S]methionine and the radiolabeled secreted proteins were concentrated and analyzed by SDS-PAGE, autoradiography, and densitometry. The data plotted in this table are an average of the arbitrary densitometry units from three experiments, and are shown as mean ± SD. The migration of p205, taken as an indication of overall protein synthesis, and the migration of collagenase are indicated on Fig. 8.

Discussion

Collagenase Gene Transcription, Activated by the $\alpha_5\beta_1$ FNR, Is Regulated by a Region of the Promoter That Contains AP1- and PEA3-Responsive Elements

In this study we have identified components of a nuclear signaling pathway, triggered by the interaction of RSF

Table II. *Expression of Gelatinase B and Collagenase in RSF Treated with FOS-OLIGO*

	FN					120FN				
	Gelatinase B	Collagenase	Gelatinase A	Gelatinase B/ gelatinase A	Collagenase/ gelatinase A	Gelatinase B	Collagenase	Gelatinase A	Gelatinase B/ gelatinase A	Collagenase/ gelatinase A
	units	*units*	*units*	*ratio*	*ratio*	*units*	*units*	*units*	*ratio*	*ratio*
FOS-OLIGO	10.8	12.0	388	0.028	0.030	485	60	424	1.14	0.14
SOF-OLIGO	3.8	3.0	158	0.023	0.018	45	7	200	0.23	0.035
Ratio (SOF/FOS)	0.35	0.25	0.41			0.09	0.11	0.47		

Proteinase-enriched conditioned medium was harvested from RSF plated for 70 h on FN or 120FN and incubated with SOF-OLIGO or FOS-OLIGO exactly as described in the legend to Fig. 8. The expression of gelatinase A, gelatinase B, and collagenase was monitored by zymography with casein or gelatin substrates incorporated in the resolving gel. The proteinase activity was used as a measure of protein expression. The expression of these proteins was quantified by densitometric analysis of photographic negatives of the zymograms, and the data are expressed as arbitrary densitometry units. Note that while the proteinases have different specific activities and, thus, a unit of collagenase has far more collagenase protein than a unit of gelatinase, the data can be used to compare the effects of particular treatments on protein levels of a particular proteinase.

with the 120-kD cell-binding fragment of FN or a function-perturbing mAb directed to the α_5 chain of the FNR, that regulates the transcription of a gene for an ECM-degrading matrix metalloproteinase, interstitial collagenase. Our data document important regulatory elements in the collagenase promoter, as well as *trans*-activating proteins responsible for this increase in collagenase gene expression.

Our experiments show that the increase in expression of −139 tkCAT in cells plated on 120FN or anti-FNR antibody, relative to FN, was higher than that of −1200 tkCAT. We also show that, overall, the basal expression of −139 tkCAT was higher than that of −1200 tkCAT. However, the induction of −1200 tkCAT by TPA was higher than that of −139 tkCAT. Our observations on the regulation of these constructs by TPA agree with previous analyses of the regulation of these chimeric promoter-reporter constructs by TPA in normal and transformed cells (Angel et al., 1987*a*,*b*; Kim et al., 1990; Lafyatis et al., 1990). A role for sequences upstream of −139 in the regulation of collagenase by TPA has also been suggested (Auble and Brinckerhoff, 1991; Chamberlain et al., 1993). Taken together, these results suggest that sequences within the −139/−67 segment of the human collagenase gene are regulated by integrin-derived signals, whereas additional sequences upstream of −139 in the human collagenase promoter are regulated by TPA.

AP1 and PEA3 sites are prominent elements in the −139/−67 segment of the collagenase promoter. Our data show that a minimal promoter construct (−97/−67) containing only these two sites (WTcol-tkCAT) is upregulated in response to 120FN and anti-FNR antibody. We have shown further that both elements are important, because minimal promoters containing mutations in either site that interfere with oligonucleotide binding in gel shift assays (Gutman and Wasylyk, 1990) were not induced in RSF plated on 120FN or anti-FNR antibody. Finally, our data suggest that AP1 is required, whereas the need for PEA3 can be obviated by the presence of multiple tandem AP1 sites. However, the presence of PEA3 was clearly required in the context of a minimal promoter (−97/−67). The requirement for the AP1 site (−72/−67) and cooperativity between the AP1 and PEA3 sites (−97/−67) have also been demonstrated in the induction of collagenase by TPA (Gutman and Wasylyk, 1990; Auble and Brinckerhoff, 1991).

Integrin-mediated Signaling Cascades Regulate Components of the AP1 Complex

The AP1 site in the collagenase promoter was first defined as a TPA-responsive element (Angel et al., 1987*b*). The dimeric transcription factor AP1 regulates the transcription of collagenase from AP1 sites either as Jun homodimers or, with higher affinity, as heterodimers of Jun and Fos family members (for review see Angel and Karin, 1992). Increases in AP1-driven transcription can be regulated by an increase in the synthesis of Fos or Jun proteins, by posttranslational modification such as phosphorylation or redox reactions, or by nuclear translocation of Fos and Jun proteins (Barber and Verma, 1987; Gius et al., 1990; Ofir et al., 1990; Abate et al., 1990, 1991; Boyle et al., 1991; Xanthoudakis and Curran, 1992; for review see Morgan and Curran, 1991). We have shown evidence that both the expression and nuclear accumulation of components of AP1 are increased in cells plated on agonistic ligands for the FNR. We saw a transient increase in c-*fos* mRNA that preceded the increase in collagenase in RSF cultured on 120FN, anti-FNR antibody, or ProNectinF, relative to that in cells cultured on FN. Furthermore, c-Fos and c-Jun proteins accumulated in the nuclei of RSF rapidly after plating on 120FN, suggesting that translocation of preexisting Fos protein is a factor in the increase in AP1-driven transcription in these experiments. These data complement the results of our experiments analyzing the transcription of the −139 tkCAT and WTcol-tkCAT constructs, because they show that the synthesis or posttranslational modification of c-Fos or c-Jun is altered as a consequence of adhesion to an agonist integrin ligand. Furthermore, incubation of cells with SOF-OLIGO, but not FOS-OLIGO, markedly decreased the integrin-induced expression of both endogenous collagenase and collagenase promoter-reporter constructs, which strongly supports the hypothesis that c-*fos*, and, by extension, AP1, is required for this increase in collagenase. Incubation of cultures with SOF-OLIGO also diminished the basal expression of collagenase in RSF cultured on FN. This may be explained by the fact that in some cases, interleukin-1 acts in an autocrine manner to increase the basal level of collagenase synthesized by synovial cells (Fini et al., 1994; Tremble, P. , and Z. Werb, unpublished observations). Interleukin-1 increases transcrip-

tion of collagenase at several sites in the promoter, one of which is the AP1 site at (or equivalent to) −72 to −66 (Lafyatis et al., 1990; Sirum-Connolly et al., 1991), so a decrease in the expression of c-Fos would also diminish the baseline synthesis of collagenase. The fold-induction of collagenase by ECM ligands was decreased, but not ablated, by incubation with SOF-OLIGO; the residual induction of collagenase in RSF plated on 120FN may be due to the generation of functional AP1 complexes through post-translational modification of existing protein, which is consistent with our observations of an increase in nuclear c-Fos protein in the nuclei of RSF cultured on 120FN within 10 min of plating. Incubation with SOF-OLIGO did not alter the fold-induction of −139 tkCAT in RSF treated with TPA. This agrees with the data of Chamberlain et al. (1993), which show that sequences upstream from −139 play a role in the regulation of collagenase by TPA. Our data comparing the regulation of −1200 tkCAT and −139 tkCAT in cultures treated with TPA are also consistent with these observations.

AP1 and PEA3 sites have been described in the promoters of many cellular and viral genes (Gutman and Wasylyk, 1991; Nerlov et al., 1991; Wasylyk et al., 1991; Yoo et al., 1991 and references therein; Campbell et al., 1991; Auble and Brinckerhoff, 1991; Buttice and Kurkinen, 1993; for review see Jones et al., 1988). They were first characterized as an activity in studies on the polyomavirus promoter (for review see Jones et al., 1988), and both were shown to be induced in response to serum, TPA, and oncogenes. In some cases, nearby AP1 and PEA3 sites are thought to work together as a TPA- and oncogene-regulated element in the promoters of genes (Gutman and Wasylyk, 1990). Members of the widely divergent Ets superfamily, which share homology primarily in their DNA-binding domain, have been shown to interact with PEA3 sites (for review see Gutman and Wasylyk, 1991). Of particular interest to the present study, Ets1 and/or Ets2 proteins have been shown to interact with oligonucleotides representing PEA3 sites from the promoters of stromelysin-1, uPA, and tissue inhibitor of metalloproteinases-1 as well as collagenase, and to *trans*-activate reporter constructs containing sequences from the polyoma early genes, c-*fos*, collagenase, and stromelysin-1 (Gutman and Wasylyk, 1990; Nerlov et al., 1991; Wasylyk et al., 1991; Campbell et al., 1991; Auble and Brinckerhoff, 1991; Buttice and Kurkinen, 1993). Likewise, AP1 sites have been identified in the promoters of tissue inhibitor of metalloproteinases-1, uPA, the 92-kD gelatinase B, and stromelysin-1 (Campbell et al., 1991; Buttice and Kurkinen, 1993; Sato and Seiki, 1993).

Taken together, the results of this study strongly suggest that a signaling pathway initiated by the integrin $\alpha_5\beta_1$ FNR regulates the activity of AP1 and, through it, the expression of collagenase in RSF. Our study also shows that the regulation or translocation of c-Fos is an obligatory intermediate in this pathway. The fact that the expression of the protein products of the stromelysin and the 92-kD gelatinase B genes is also regulated by this integrin-initiated pathway bolsters the concept that activation of this pathway may be a particularly important consequence of signaling through the FNR. The fact that these genes are all involved in the regulation of ECM remodeling suggests that an important role for matrix receptors is to monitor the integrity of the ECM and to promote its remodeling in response to specific signals. In this study the inductive signals had two things in common: they included (120FN) or mimicked (anti-FNR antibody, ProNectinF) the RGD central cell-binding domain of FN and were able to bind the $\alpha_5\beta_1$ FNR. However, they did not include or mimic other cell-binding regions present in intact FN, such as the carboxyl-terminal heparin-binding domain or the CS-1 binding site. Thus, the cell appears to activate the signaling pathway that leads to the expression of matrix remodeling genes when it receives the signal from the central cell-binding domain alone, via the FNR, but not when it receives the complete set of signals from intact FN, which interacts with both integrin and nonintegrin cell surface receptors. Under physiologic conditions, similar inductive ligands may be present at sites of wounding or inflammation, where FN fragments and other inductive modifications of ECM are likely to be generated (see Tremble et al., 1994; Huhtala et al., 1995).

Signals Initiated by Integrins and Receptors for Growth-Regulatory Factors Intersect at the Level of Transcription

Reports from other systems suggest that changes in the amount or activity of components of AP1 correlate either with adhesion to specific ECMs or with changes in the cellular cytoskeleton. In T lymphocytes, the expression of c-*fos* and the AP1-driven transcription of interleukin-1 is upregulated when both CD3 and the FNR are occupied with anti-receptor antibodies or ligand (Yamada et al., 1991). c-*fos* mRNA is also increased in monocytes adhering to FN (Shaw et al., 1990) and in adhering, growth-arrested fibroblasts (Dike and Farmer, 1988). Destabilization of actin-supported cytoarchitecture has been shown to lead to an increase in c-*fos* mRNA in some cell systems (Zambetti et al., 1991) and to an accumulation of c-*jun* mRNA in others (Botteri et al., 1990).

Other transcriptional response elements in genes have been shown to respond to changes in cell adhesion, supporting the concept that adhesion to specific ligands has different consequences for cellular function than adherence alone. A group of genes that appear to be regulated with changes in cell adhesion has been identified in monocytes; some of these genes encode cytokines, and others encode transcription factors or their inhibitors. A number of these monocyte adherence genes are regulated by cell adhesion alone (i.e., to plastic), whereas others require the engagement of specific $\beta1$ integrin receptors (for review see Juliano and Haskill, 1993). The promoters of monocyte adherence genes contain consensus sites for transcription factors in the NF-κB, c-myb, and helix-loop-helix families, suggesting that these elements may respond to integrin-derived signals in monocytes. Indeed, in adhering monocytes, NF-κB localizes in the nucleus (Griffin et al., 1989). In mammary epithelial cells, the expression of milk proteins also responds to cues provided by cell-ECM interactions (Streuli et al., 1991). In culture, the transcription of β-casein, a marker for tissue-specific gene expression in mammary epithelial cells, is upregulated in response to prolactin only in the context of an appropriate

ECM substrate. An element in the promoter of the β-casein gene, BCE1, that regulates the expression of β-casein in response to concurrent integrin and hormonal stimulation has been identified (Schmidhauser et al., 1990), providing yet another example of the convergence of integrin and hormonally regulated transduction pathways.

Signaling pathways initiated by diverse stimuli can regulate the expression and/or activity of AP1. This suggests a mechanism by which signaling pathways initiated by integrins intersect with pathways initiated by other receptors. In other systems the cytoplasmic machinery involved in activation of AP1 is beginning to be characterized. The phosphorylation of serine 63 and 73 in the *trans*-activating domain of c-Jun and the dephosphorylation of critical residues in its DNA-binding domain are modulated by a protein kinase cascade that may include the cytosolic oncoproteins Ha-ras, v-src, and Raf1 (Smeal et al., 1992). Studies in which individual kinases with mutations that result in proteins with a dominant-negative phenotype are overexpressed have established that Raf1 is downstream from protein kinase C, Ha-ras, and v-src (for review see Rapp, 1991; Kolch et al., 1993; Bruder et al., 1992; Qureshi et al., 1992) but acts upstream of MAP kinase-kinase and MAP kinase (Pulverer et al., 1993; Kyriakis et al., 1992). Thus, there are some common features in the protein kinase cascades regulating AP1, which are initiated by diverse stimuli such as phorbol esters, serum, cytoplasmic oncogenes, and ultraviolet light (Wasylyk et al., 1989; Bruder et al., 1992; Radler-Pohl et al., 1993). Working backwards from the regulation of AP1 should help clarify the cytoplasmic signals initiated by integrins that play a role in regulating gene expression.

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