The Mitogen-inducible Fn14 Gene Encodes a Type I Transmembrane Protein that Modulates Fibroblast Adhesion and Migration*  

(Received for publication, May 28, 1999, and in revised form, July 23, 1999)  

Rachel L. Meighan-Mantha‡, Debbie K. W. Hsu‡, Yan Guo‡, Sharron A. N. Brown‡, Sheau-Line Y. Feng‡, Kimberly A. Peifley‡, Gregory F. Alberts‡, Neal G. Copeland‡, Debra J. Gilbert‡, Nancy A. Jenkins‡, Christine M. Richards‡, and Jeffrey A. Winkles‡‡**  

From the ‡Department of Vascular Biology, Holland Laboratory, American Red Cross, Rockville, Maryland 20855, the §Mammalian Genetics Laboratory, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702, and the ¶Department of Biochemistry and Molecular Biology and the Institute for Biomedical Sciences, George Washington University Medical Center, Washington, D. C. 20037.  

The binding of polypeptide growth factors to their appropriate cell surface transmembrane receptors triggers numerous biochemical responses, including the transcriptional activation of specific genes. We have used a differential display approach to identify fibroblast growth factor-1-inducible genes in murine NIH 3T3 cells. Here, we report that the fibroblast growth factor-inducible-14 (Fn14) gene is a growth factor-regulated, immediate-early response gene expressed in a developmentally and adult tissue-specific manner in vivo. This gene, located on mouse chromosome 17, is predicted to encode an 129-amino acid type Ia membrane protein with no significant sequence similarity to any known protein. We have used two experimental approaches, direct fluorescence microscopy and immunoprecipitation analysis of biotinylated cell surface proteins, to demonstrate that Fn14 is located on the plasma membrane. To examine the biological consequences of constitutive Fn14 expression, we isolated NIH 3T3 cell lines expressing variable levels of epitope-tagged Fn14 and analyzed their phenotypic properties in vitro. These experiments revealed that Fn14 expression decreased cellular adhesion to the extracellular matrix proteins fibronectin and vitronectin and also reduced serum-stimulated cell growth and migration. These results indicate that Fn14 is a novel plasma membrane-spanning molecule that may play a role in cell-matrix interactions.

Complex cellular processes such as proliferation, migration, differentiation, and apoptosis are regulated in part by a diverse group of molecules known as polypeptide growth factors. These factors act by binding and thereby activating specific transmembrane receptor tyrosine kinases. The activation of cell surface receptors by polypeptide ligands triggers downstream intracellular events, including the stimulation of protein phosphorylation cascades and the transcriptional activation of numerous genes (1, 2). Many mitogen-inducible genes have been identified, and they encode a diverse group of proteins including transcription factors, protein kinases and phosphatases, cell cycle regulators, and cytoskeletal and extracellular matrix proteins (2, 3). A recent study using cDNA microarray technology has demonstrated that >500 genes are transcriptionally activated after serum stimulation of quiescent human fibroblasts and that a subset of these genes encode proteins implicated in the wound healing process in vivo (3).

Our laboratory has been studying fibroblast growth factor-1 (FGF-1)-regulated gene expression in murine NIH 3T3 cells. FGF-1 (also referred to as acidic FGF) is one of the most extensively characterized members of the FGF family of heparin-binding proteins (4–6). It is a potent mitogenic, chemotactic, angiogenic, and neurotrophic factor both in vitro and in vivo. These cellular responses are mediated via high affinity binding to a family of related membrane-spanning tyrosine kinase receptors (4–6). We have shown by Northern blot hybridization analysis that FGF-1 stimulation of quiescent NIH 3T3 cells induces the expression of several previously described serum-regulated genes; e.g. c-fos, c-jun, c-myc, thrombospondin-1, and ornithine decarboxylase (7, 8). In addition, we have used a differential display approach to isolate several cDNA clones representing previously unreported mitogen-inducible genes (9). Genes identified to date using this strategy include those encoding an aldose reductase-related protein (10, 11), a member of the pol family of serine/threonine protein kinases (12, 13), and a member of the transcriptional enhancer factor-1 family of DNA-binding proteins (14).

In this paper, we report that the murine Fn14 gene is a growth factor- and phorbol ester-regulated immediate-early response gene encoding a novel type Iα transmembrane protein, with an amino-terminal signal peptide, a 53-aa ectodomain, a single hydrophobic transmembrane domain, and a 28-aa carboxyl-terminal cytoplasmic domain. Analysis of transfected NIH 3T3 cell lines that constitutively express the Fn14

* This work was supported in part by National Institutes of Health Grant HL-39727 (to J. A. W.) and by the NCI, National Institutes of Health, Department of Health and Human Services, under contract with ABL (to N. G. C. and N. A. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom all correspondence should be addressed: Dept. of Vascular Biology, Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855. Tel.: 301-738-0806; Fax: 301-738-0465; E-mail: winkles@usa.redcross.org.

† The abbreviations used are: FGF, fibroblast growth factor; aa, amino acid(s); EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; Fn14, FGF-inducible 14; GST, glutathione S-transferase; HA, hemagglutinin; IGF, insulin-like growth factor; IGF-1, insulin-like growth factor-1; KDR, kinase insert domain receptor; MAPK, mitogen-activated protein kinase; Mcl, myc; m-RAC, minisatellite RAC; PMA, phorbol myristate acetate; RACE, rapid amplification of cDNA ends; RFLP, restriction fragment length polymorphism; TGF, transforming growth factor; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation assay; HRP, horseradish peroxidase; nt, nucleotide(s); kb, kilobase(s); bp, base pair(s); BSA, bovine serum albumin; TBST, Tris-buffered saline with Tween 20.

This paper is available on line at http://www.jbc.org
protein indicate that it may be involved in the regulation of cellular adhesion, growth, and migration.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Murine NIH 3T3 fibroblasts (American Type Culture Collection) were grown, expanded, and serum-starved as described (15). Serum-starved cells were then either left untreated or treated with one of the following: 10 ng/ml human recombinant TGF-β (kind gift of W. Burgess, Holland Laboratory) in combination with 5 units/ml heparin (Upjohn), 10 ng/ml human recombinant TGF-β (Bachem), 10 ng/ml human recombinant PDGF-BB (Genzyme), 2 ng/ml human recombinant TGF-β (R&D Systems), 20 ng/ml human recombinant IGF-1 (Bachem), 10% calf serum (HyClone), or 30 ng/ml PMA (Sigma). In some experiments, NIH 3T3 cells were treated with 2 µg/ml actinomycin D (Calbiochem) or 10 µg/ml cycloheximide (Sigma).

**RNA Isolation and Differential Display**—Cells were harvested by trypsin/EDTA treatment, and total RNA was isolated using RNA Stat-60 (Tel-Test) per the manufacturer’s instructions. RNA from newborn or adult FVB/N mice (Taconic Farms) were obtained, and RNA was isolated as above after the samples were initially homogenized using a Tissumizer (Tekmar). RNA concentrations were calculated by measuring UV light absorbance at 260 nm. RNA (1 µg) from quiescent or PMA-treated NIH 3T3 cells was converted to cDNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and random hexamer primers (Roche Molecular Biochemicals) as described (16). PCR assays were performed using a degenerate sense protein kinase domain oligonucleotide primer (12) and a degenerate anti sense leucine zipper domain oligonucleotide primer (17). An equivalent aliquot of each amplification mixture was subjected to electrophoresis in a 2% agarose gel, and DNA was visualized by ethidium bromide staining. An ~230-bp DNA fragment was excised, recovered using the freeze-squeeze method (18), reamplified, and ligated into the vector pCRII using a T/A cloning kit (Invitrogen).

**cDNA Library Screening**—A mouse Balb/c 3T3 cell cDNA library (kind gift of T. Lanahan, Johns Hopkins University School of Medicine, Baltimore, MD) was screened with the PCR-derived Fn14 cDNA fragment. Several cDNA clones of the Fn14 cDNA fragment were identified with [α-32P]dCTP (3000 Ci/mmol, Amersham Pharmacia Biotech) using a random primer DNA labeling kit (Roche Molecular Biochemicals). Approximately 2 × 10^5 plaque were plated at a density of 2 × 10^4 plaque-forming units/150-mm dish using Escherichia coli C600 Hfl as the host. Duplicate plaque lifts (Colonies/Plaque screen, DuPont) were pre-hybridized, hybridized, washed and exposed to Kodak X-Omat AR film as described (14). Five positive plaques were plated, purified and amplified on E. coli C600 Hfl cells. Plasmids were excised from the plaque clones using XL-1 Blue cells and R408 helper phage (Stratagene) as described (19) and designated pBluescript/Fn14.

**5'-RACE Assays**—A cDNA fragment representing the 5' region of Fn14 mRNA was identified by PCR using mouse heart 5'-RACE Ready cDNA library (Clontech) and random primers. cDNA hybridization probes were used to screen a mouse heart 5'-RACE-Ready cDNA library using a T/A cloning kit (Invitrogen). A pBluescript/Fn14 plasmid containing the Fn14 cDNA sequence without the 3' untranslated region was constructed and linearized using the appropriate enzymes, and sense or antisense riboprobes were transcribed in vitro using T3 or T7 RNA Polymerase (Roche Molecular Biochemicals), [α-32P]UTP (1000 Ci/mmol, NEN Life Science Products) and reagents included in the SureScript II in situ hybridization kit (Novagen). Riboprobes were subjected to alkaline hydrolysis to yield an average length of ~100 nt, as verified by denaturing PAGE. Pre-hybridization and hybridization conditions were per the manufacturer’s instructions except that the hybridization step was performed at 65 °C. Post-hybridization washes were once with 2× SSC for 5 min at room temperature, once with 2× SSC for 30 min at 65 °C, once with 2× SSC for 1 h at 65 °C, once with 0.5× SSC containing 50% formamide for 30 min at 50 °C, twice with 1× SSC for 30 min at 50 °C, and once with 0.1× SSC for 30 min at 65 °C. Slides were dipped in Kodak NTB-2 emulsion, counterstained with hematoxylin and eosin, and mounted.

**Expression of Recombinant Fn14 in Bacterial Cells and Generation of Fn14 Antiserum**—Recombinant Fn14 was produced by expression of the plasmid E. coli as a GST fusion protein. The expression plasmid was constructed by ligation of an EcoRI-XhoI restriction fragment of pBluescript/Fn14 into the same sites located in the polyclinker of pGEX-KG (kind gift of R. Friesel, Maine Medical Center Research Institute, Portland ME). DNA sequence analysis was performed to confirm the construct. E. coli HB101 cells (Life Technologies, Inc.) that have been transformed with this plasmid were cultured overnight at room temperature in Luria broth containing 100 µg/ml ampicillin (Sigma). Cells were diluted 1:10 in Luria broth, grown for 5 h at room temperature, induced with 0.1 µM isopropyl-β-D-thiogalactopyranoside (Life Technologies, Inc.) for 3 h, and pelleted by centrifugation. Cells were resuspended by repeated pipetting in STE buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) containing 1 mg/ml lysozyme (Sigma) and incubated for 20 min on ice. Dithiothreitol (Sigma) was added to a final concentration of 5 mM, the lysate was vortexed, N-laurylsarcosine (Sigma) was added to a final concentration of 1.5%, and again the lysate was vortexed. After mild sonication, the lysate was clarified by centrifugation, and Triton X-100 was added to the supernatant at a 2% final concentration. The supernatant was then incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) for 30 min at 4 °C with end-over-end mixing. The beads were collected by brief centrifugation and washed five times with PBS containing 1% Triton X-100. To prepare antigen for rabbit immunization, GST-Fn14 was released from glutathione-Sepharose beads by the addition of 100 mM Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and dialyzed against PBS. A New Zealand White rabbit was injected with ~0.5 mg of the antigen in complete Freund’s adjuvant.
Mitogen Regulation of Fn14 Gene Expression

adjuvant (Calbiochem) and boosted five times with ~0.3 mg of the antigen in incomplete adjuvant (Calbiochem). Crude serum was used for the Western blot experiments.

**Construction of the Fn14-GFP and Fn14-HA Eukaryotic Expression Plasmids**—The plasmid pEGFP-N3/Fn14, which encodes Fn14 with a carboxy-terminal biotinylation signal (24), was isolated for 30 min at 50 °C. A 1:10 split was performed using phBluescript/Fn14 as the template, a sense primer containing a 5′ BamHI restriction site followed by Fn14 nucleotides −12 to +12, an antisense primer representing Fn14 nucleotides 380–399 and Taq polymerase (Life Technologies, Inc.). The DNA product was isolated and then ligated into pCR2.1 using a T/A cloning kit (Invitrogen). A BamHI/EcoRI fragment representing the Fn14 coding sequence was filled-in using T4 DNA polymerase and cloned into the expression vector pEGFP-N3 (CLONTECH). The plasmid pcDNAneo/Fn14-HA, which encodes Fn14 with a carboxy-terminal-terminal influenza HA epitope tag (25), was constructed by first subcloning an EcoRI/Xhol fragment of phBluescript/Fn14 into the EcoRI and HindIII cloning sites of the expression vector pEGFP-N3 (CLONTECH). The plasmid pEGFP-N3/Fn14, which encodes Fn14 with a carboxy-terminal-terminal influenza HA epitope tag (25), was constructed by first subcloning an EcoRI/Xhol fragment of pEGFP-N3 into the EcoRI and HindIII cloning sites of the expression vector pMEXneo (26). DNA sequence analysis of transfected cells, proteins were transferred to Protran nitrocellulose membranes (Schleicher & Schuell) by electroblotting. The membrane was blocked for 1 h at room temperature in TBST containing 3% BSA and incubated in TBST containing 3% BSA and 200 ng/ml streptavidin-HRP (Pierce) for 30 min. Biotinylated proteins were detected using the Amersham Pharmacia Biotech ECL system.

**Identification of an FGF-1-inducible Gene by mRNA Differential Display**—We have described previously a differential display approach that employs random hexamer-primed cDNA templates, PCR oligonucleotide primers designed to anneal with sequences encoding motifs found in particular protein structural domains, and agarose gel electrophoresis (9). In the present experiment, RNA was isolated from either quiescent NIH 3T3 cells or cells that had been treated with both FGF-1 and cycloheximide for 2 h. This RNA was converted to DNA using the enzyme reverse transcriptase, and PCR assays were performed using sense protein kinase domain and antisense leucine zipper domain oligonucleotide primers. Amplification products were displayed using agarose gel electrophoresis and ethidium bromide staining. An ~230-bp DNA fragment was carried out using EZ-Link Sulfo-NHS-LC-Biotin (Pierce) per the manufacturer’s instructions. The cells were lysed in modified 1× RIPA buffer (50 mM Tris/Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture; 0.1 mg/ml aprotinin and 0.1 mg/ml leupeptin) for 2 h at 4 °C and clarified by centrifugation.

**RESULTS**

**RESULTS**
Mitogen Regulation of Fn14 Gene Expression

FIG. 1. Murine Fn14 nucleotide sequence and deduced amino acid sequence. Numbers to the left refer to the first amino acids on the lines, and the numbers to the right refer to the last nucleotides on the lines. The nucleotide sequence obtained from a 5′-RACE-derived cDNA clone is in boldface type. The solid line below amino acids 1–27 indicates the predicted signal peptide sequence, and the boxed amino acid stretch indicates the predicted transmembrane domain. In the 3′-untranslated region, the stop codon is denoted by an asterisk, a putative mRNA destabilization sequence motif is underlined, and the polyadenylation signal is boxed.

was amplified to a greater degree when cDNA representing the RNA isolated from cells treated with FGF-1 and cycloheximide was used as template (data not shown). This DNA fragment was isolated, cloned, and used as a probe in a preliminary Northern blot hybridization experiment to confirm that it did indeed represent an FGF-1-inducible gene. This gene was named the FGF-inducible 14 (Fn14) gene, because it was predicted to encode an 14-kDa protein (see below).

**Fn14 cDNA Sequence Analysis**—A mouse fibroblast cDNA library was then screened with the differential display-derived cDNA fragment in order to isolate longer cDNA clones. Both strands of the longest cDNA insert (−950 bp) were sequenced by the dideoxynucleotide chain termination method. The nucleotide sequence contained a 12-nt 5′-untranslated region, and a 550-nt 3′-untranslated region, a 691-nt open-reading frame, and a 550-nt 3′-untranslated region. The nucleotide sequence contained a 12-nt 5′-untranslated region, and a 550-nt 3′-untranslated region.

The Fn14 gene is predicted to encode a protein of 129 aa with a molecular mass of 13,637 daltons and an isoelectric point of 8.18. The protein is proline-rich (9.3%) and contains eight cysteines but is devoid of asparagine or tyrosine residues.

Computer analysis of the predicted Fn14 amino acid sequence revealed no significant degree of sequence identity between Fn14 and other known proteins. However, we have noted that the Fn14 transmembrane domain has some sequence similarity to the transmembrane region found in the syndecan family of integral plasma membrane proteins. Specifically, alignment of the Fn14 transmembrane sequence to the transmembrane sequences of the mouse, rat, and human syndecan proteins revealed that four amino acids are conserved between Fn14 and all the syndecans, while five more amino acids are conserved between Fn14 and one or more of the syndecans. These nine conserved Fn14 amino acids are shown in boldface type: (I)-L-(G-G)-A-L-S-(L)-V-L-V-L-A-(L-V)-S-S-(F-L-V)-W.

Of special interest is conservation of the double glycine, since the transmembrane region of the syndecans contains this motif and in general, glycine residues are found infrequently in the transmembrane region of the syndecans. These nine conserved Fn14 amino acids are shown in boldface type: (I)-L-(G-G)-A-L-S-(L)-V-L-V-L-A-(L-V)-S-S-(F-L-V)-W.
Mitogen Regulation of Fn14 Gene Expression

We first investigated the kinetics of Fn14 mRNA accumulation following FGF-1 stimulation of NIH 3T3 cell growth by Northern blot hybridization analysis. A single Fn14 transcript of ~1.2-kb in size was detected in FGF-1-treated cells (Fig. 2A). Increased Fn14 mRNA levels were first evident at 1 h after FGF-1 addition, and maximal levels were present at 4 h. The effect of the RNA synthesis inhibitor actinomycin D and the protein synthesis inhibitor cycloheximide on FGF-1 induction of Fn14 mRNA levels was also examined. Actinomycin D co-treatment inhibited FGF-1 induction of Fn14 mRNA (Fig. 2B); in contrast, cycloheximide co-treatment resulted in Fn14 mRNA superinduction (Fig. 2C). Cycloheximide treatment alone also increased Fn14 mRNA levels. Taken together, these results indicate that Fn14 is an FGF-1-inducible, transcription-translationally activated immediate-early response gene.

We then determined whether the FGF-1-related mitogen FGF-2 (also referred to as basic FGF), PDGF-BB, TGF-β1, EGF, IGF-1, calf serum, or PMA could also increase Fn14 mRNA levels. FGF-1, FGF-2, PDGF-BB, TGF-β1, EGF, IGF-1, calf serum, and PMA could each induce Fn14 mRNA expression to a similar extent (Fig. 2D). In contrast, TGF-β1 or EGF treatment had only a slight stimulatory effect, while IGF-1 had no detectable activity.

Regulation of Fn14 Protein Expression in NIH 3T3 Cells—We next investigated whether mitogenic stimulation of quiescent NIH 3T3 cells resulted in elevated Fn14 expression. We first obtained Fn14 polyclonal antiserum by immunizing rabbits with recombinant GST-Fn14 fusion protein purified from bacterial cultures. Initial Western blot experiments indicated that this antiserum specifically recognized recombinant Fn14 protein expressed in either bacterial or insect cell systems (data not shown). To analyze Fn14 expression levels in growth factor-stimulated cells, serum-starved cells were either
left untreated or treated with FGF-2 for different lengths of time and Western blot analysis was conducted using the Fn14 antiserum. A major immunoreactive protein of ~22 kDa was detected in FGF-2-treated cells (Fig. 4). FGF-2 stimulation increased the expression level of this protein, with maximal levels present at 12 h after mitogen addition. The apparent molecular mass of this immunoreactive protein is approximately twice the predicted size of the mature Fn14 protein (minus signal peptide); however, we have concluded that this protein is Fn14 based on several experimental findings (see “Discussion”).

**Fn14 mRNA Expression in Mouse Embryos and Tissues—** The pattern of Fn14 mRNA expression during mouse development was investigated. A blot containing RNA isolated from four different developmental time-points was obtained, and Fn14 mRNA levels were examined by Northern hybridization analysis. Fn14 mRNA expression was detected at all of the time points examined (Fig. 5A). Maximal levels of expression were detected at 7.5 days post-coitum; however, this RNA sample was isolated from both the developing embryo and the surrounding extra-embryonic and maternal tissues. To identify the precise sites of Fn14 mRNA expression, we performed *in situ* hybridization analysis on serial sections from 8.5-day post-coitum mouse embryos sectioned in utero. Fn14 transcripts were detected primarily in the maternal decidual tissue nearest the ectoplacental (mesometrial) pole (Fig. 5B).

The tissue distribution of Fn14 mRNA was evaluated by Northern blot analysis using RNA isolated from tissues obtained from either newborn or adult mice. In the newborn animals, Fn14 transcripts were expressed at a relatively high level in all six tissues examined (Fig. 6A). In adult mice, Fn14 mRNA was expressed at the highest level in heart and ovary and at an intermediate level in kidney, lung and skin (Fig. 6B). Fn14 mRNA was expressed at a relatively low level in the other seven adult tissues examined. Thus, the Fn14 gene is expressed in a developmental stage- and tissue-specific manner in vivo.

**Fn14 Subcellular Localization Studies—** Fn14 was transiently expressed as a EGFP fusion protein in NIH 3T3 cells in order to determine its subcellular location. When EGFP itself is expressed in these cells, it is primarily found in the cytoplasm (Fig. 7A). In contrast, the Fn14-EGFP fusion protein is localized to the plasma membrane and to the trans-Golgi network near the nuclear membrane (Fig. 7, B and C). At the cell surface, Fn14-EGFP is especially prominent in thin membrane extensions resembling microspikes or filopodia (Fig. 7C). In additional experiments, Fn14 containing distinct amino- and carboxyl-terminal epitope tags was expressed in transfected NIH 3T3 cells and localization was assayed by immunofluorescence microscopy of permeabilized or non-permeabilized cells. We found that Fn14 was oriented with its amino terminus located outside of the cell (data not shown), and this result is consistent with the orientation predicted by computer analysis of the Fn14 primary sequence.

We confirmed that Fn14 was present on the plasma membrane using a biochemical approach. First, the expression plasmid pcDNAIneo/Fn14-HA, which encodes a Fn14 protein containing a carboxyl-terminal HA epitope tag (25), was con-
3T3 Cells—We compared the properties of the control (vector-induced) and NIH 3T3 cell lines transfected or stably transfected with the pEGFP-N3 vector (panel A) or the pEGFP-N3/Fn14 plasmid (panels B and C) were processed at 24 h after transfection and viewed using fluorescence microscopy. Original magnification, ×600.

Biological Effects of Fn14-HA Expression in Transfected NIH 3T3 Cells—We compared the properties of the control (vector-induced) or Fn14-HA-expressing clonal cell lines described above using several different assays in order to gain insight into the possible biological functions of the Fn14 protein. First, we determined whether constitutive Fn14 expression had an effect on NIH 3T3 cell adhesion to various extracellular matrix proteins. Neither the control nor the three experimental cell lines were able to adhere to collagen I or collagen IV; furthermore, these cell lines displayed only weak adherence to laminin. In comparison to the control V5 line, the two experimental lines expressing the highest levels of the Fn14-HA protein (lines 11 and 10) exhibited decreased adhesion to fibronectin, while all three experimental cell lines (lines 3, 11, and 10) exhibited decreased adhesion to vitronectin. Adhesion of the Fn14-HA 10 cell line on fibronectin and vitronectin was reduced by ~42% and ~74%, respectively. We subsequently performed an additional set of adhesion assays in which the control V5 and the Fn14-HA 10 cell lines were plated onto increasing concentrations of vitronectin. For both cell lines, the extent of cell adhesion increased in a dose-dependent manner; however, in comparison to the control cell line, the Fn14-HA-expressing cell line exhibited decreased adhesion at all of the vitronectin concentrations tested (Fig. 9B). We have also observed that when the V5 and Fn14-HA 10 cells are plated on vitronectin and examined ~4 h later, the V5 cells are spread out and display a typical fibroblast morphology while the Fn14-HA-expressing cells are more spherical in shape (data not shown).

Second, since cell-extracellular matrix interactions play a key role in cellular proliferation and migration, we determined whether constitutive Fn14 expression altered serum-stimulated NIH 3T3 cell growth or motility. When the growth prop-
erties of the control V5 cell line and the Fn14-HA 10 cell line were compared in serum-containing medium using a colorimetric assay, we observed that the Fn14-HA-expressing cell line had a statistically significant decrease in growth rate. In comparison to the V5 cells, growth of the Fn14-HA 10 cells was reduced by 60% after 4 days of culture (Fig. 10A). Similar results were obtained when cellular proliferation was measured by directly counting viable cells (data not shown). These same two cell lines were then used in cellular migration assays. Each cell line was resuspended in serum-free medium and plated in the upper compartment of Transwell inserts, and their migration through a porous membrane in response to either serum-free medium or serum-containing medium was measured. When serum-free medium was present in both the upper and lower compartments, no cellular migration occurred (data not shown). When serum-containing medium was placed in the lower compartment, both the control and experimental cells exhibited chemotactic migration; however, migration of the Fn14-HA-expressing cell line was ~40% of that observed with the control cell line (Fig. 10B).

DISCUSSION

The addition of serum or polypeptide growth factors to quiescent fibroblast cultures initiates an intracellular signal transduction cascade that promotes the transcriptional activation of numerous cellular genes (1–3). These genes are generally classified as either immediate-early, delayed-early, or late response genes, and they encode proteins with diverse functions (2, 3). We have used a differential display approach to identify FGF-1-inducible genes in NIH 3T3 cells (9), and here we describe the cloning, chromosomal location, and expression pattern of the Fn14 gene. We also report the biological effects of constitutive Fn14 expression in transfected cells.

The original Fn14 cDNA was isolated by differential display using degenerate sense and antisense oligonucleotide primers designed to anneal to DNA sequences encoding conserved amino acid motifs found in protein kinase domains and leucine zipper domains, respectively. We found that neither of these structural domains were present in the predicted Fn14 sequence. This result was not unexpected since comparison of the PCR primer sequences with the corresponding regions of the Fn14 cDNA sequence indicated that both primers annealed to regions of relatively low sequence identity. The Fn14 gene is
predicted to encode a type Ia transmembrane protein containing a 27-aa signal peptide, a 53-aa extracellular domain, a 21-aa transmembrane domain, and a 28-aa cytoplasmic domain. The mature 102-aa Fn14 polypeptide is predicted to have a molecular mass of ~10.8 kDa. Sequence motifs present in the Fn14 sequence include a cluster of charged amino acids following the predicted membrane-spanning domain and a L-I dipeptide in the cytoplasmic tail. The first motif may function as a stop-transfer signal during membrane insertion, and the second motif could serve as an endosomal targeting signal (33–35).

The deduced Fn14 protein sequence was compared with the GenBank sequence data base, and no significant sequence identity was found between Fn14 and known proteins. However, a pattern search using Fn14 transmembrane domain residues revealed some sequence similarity to the single transmembrane domain found in the syndecan family of heparan sulfate proteoglycans (38, 39). The highest overall amino acid sequence identity in this domain, ~33%, was between Fn14 and murine syndecan-1. Of particular interest is that (i) Fn14 and all four syndecan family members contain a G-G dipeptide motif in their transmembrane domains, even though glycine residues are not generally found in membrane-spanning sequences (36) and (ii) Fn14 contains a pattern of bulky and small side chain residues that is similar, but not identical, to the pattern found in the syndecan transmembrane domain (37). It has been demonstrated by site-specific mutagenesis that this pattern is critical for the formation of noncovalent, SDS-resistant syndecan-3 dimers and oligomers (37). It is presently unknown whether the Fn14 protein, like the syndecans, can self-associate, but it does not migrate at its predicted molecular weight when analyzed by SDS-PAGE (see below).

The Fn14 gene is located on mouse chromosome 17, in the middle of the T-locus. We have compared our interspecific map of chromosome 17 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (Mouse Genome Data Base). Fn14 mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus. The proximal region of mouse chromosome 17 shares a region of homology with human chromosomes 6 and 16 (summarized in Fig. 2). In particular, E4fl has been assigned to human 16p13.3-p13.12. The tight linkage between E4fl and Fn14 in mouse suggests that the human homolog of Fn14 will map to this same chromosomal location. Consistent with this possibility, a recent search of the GenBank database reveals the murine Fn14 nucleotide sequence reveals a significant degree of sequence relatedness between Fn14 and human chromosome 16p13.3 genomic DNA (GenBank accession no. AC004643).

The Fn14 gene can be classified as a growth factor-inducible, immediate-early response gene *in vitro* that is expressed in a developmental stage- and tissue-specific manner *in vivo*. FGF-1 stimulation of quiescent fibroblasts rapidly increases Fn14 mRNA levels, with peak expression detected at 4 h. It is likely that this response is due, at least in part, to transcriptional activation of the Fn14 gene since Fn14 mRNA accumulation does not occur in the presence of an RNA synthesis inhibitor. Fn14 mRNA levels remain elevated for a significant period of time after mitogen addition, suggesting that Fn14 gene transcription is somewhat sustained and/or Fn14 mRNA is relatively stable. In regard to this second possibility, we have noted that the Fn14 mRNA 3'-untranslated region contains one copy of an AU-rich motif implicated in rapid mRNA decay (30), and therefore this transcript may have a short half-life. However, nuclear run-on assays and mRNA stability measurements are required in order to determine the precise molecular basis for the Fn14 mRNA temporal expression pattern that is observed. FGF-1 induces Fn14 mRNA levels in the presence of a protein synthesis inhibitor; thus, Fn14 gene activation does not require the *de novo* synthesis of intermediary proteins. The simultaneous addition of FGF-1 and cycloheximide promoted Fn14 mRNA superinduction and, in addition, cycloheximide treatment alone induced Fn14 mRNA levels. These cycloheximide effects are likely to occur because the drug is preventing the synthesis of labile proteins required for Fn14 transcriptional repression and/or Fn14 mRNA decay. We found that, like the majority of the immediate-early genes identified to date, the Fn14 gene can be induced by various polypeptide growth factors as well as by PMA, a tumor-promoting phorbol ester that activates protein kinase C. Finally, Fn14 transcripts are expressed in mice at a relatively high level in the maternal decidual tissue of the developing embryo, in many of the major organs of newborn animals, and in the adult heart, kidney, lung, ovary, and skin.

We generated Fn14 polyclonal antiserum and then performed Western blot analysis to investigate whether the mitogenic stimulation of NIH 3T3 cells increased Fn14 protein expression. FGF-2 treatment of quiescent cell cultures induced the accumulation of a ~22-kDa immunoreactive protein, and the temporal expression kinetics observed were consistent with the kinetics of Fn14 mRNA expression in FGF-1-treated cells. Although this immunoreactive protein migrated at an apparent molecular mass twice the predicted mass of mature Fn14 (~10.8 kDa), we believe it is likely to be Fn14 for two reasons. First, when Fn14 cDNA is expressed in baculovirus-infected insect cells, the 129-aa protein is processed and mature Fn14 migrates an apparent molecular mass of ~23 kDa (data not shown). Second, when an HA epitope-tagged Fn14 protein predicted to be ~11.9 kDa in size is expressed in transfected NIH 3T3 cells, the major Fn14-HA protein species detected is ~23 kDa in size (see Fig. 8). Fn14 may be migrating at a higher apparent molecular mass when analyzed by SDS-PAGE due to post-translational modifications, self-association into reducing agent/SDS-resistant dimers or an amino acid composition (*e.g.* high proline content) that causes the protein to resist denaturation or to bind SDS poorly. In regard to the first possibility, this explanation is unlikely because (i) there are no consensus sequence motifs for N-glycosylation or glycosaminoglycan attachment in the predicted Fn14 protein, (ii) the major Fn14 protein synthesized in an *in vitro* transcription/translation system also migrates at ~22 kDa (data not shown), and (iii) full-length Fn14 expressed in bacterial cells also migrates at a higher apparent molecular mass than predicted from the cDNA sequence when it is analyzed by SDS-PAGE (data not shown).

In regard to the second possibility, although it is possible that Fn14 could be forming dimers, especially when one considers that the Fn14 transmembrane domain has some sequence identity to the region of the transmembrane domain implicated in syndecan-3 self-association (37), we have never detected Fn14 migrating at a molecular mass indicative of a monomeric molecule or of oligomeric complexes. Thus, we presently favor the third possibility, anomalous migration due to amino acid composition, as the likely explanation for the higher than predicted apparent molecular mass of the Fn14 protein.

Two independent experimental approaches were used to demonstrate that Fn14 is a plasma membrane protein. Fluorescence microscopy analysis of Fn14-EGFP localization in transfected NIH 3T3 cells indicated that Fn14 was concentrated in the trans-Golgi network (probably due to inefficient transport of overexpressed protein through the secretory pathway) and in areas along the cell periphery that could represent regions of cell-substratum attachment; however, additional colocalization experiments are required to confirm that these are
in fact focal adhesion sites. Fn14 was also present on the cell surface in thin membrane protrusions resembling actin microspikes or filopodia, which are believed to act as sensory structures that play a role in the control of cell growth and migration (40–42). Fn14 was also localized to the plasma membrane of a Fn14-HA-expressing cell line using cell surface biotinylation followed by immunoprecipitation and Western blot analysis. NIH 3T3 stable cell lines expressing HA epitope-tagged Fn14 were isolated in order to examine the biological consequences of constitutive Fn14 expression. We are aware that a limitation of this experimental approach is that Fn14 may exhibit abnormal behavior when overexpressed in cells; therefore, we have analyzed several independent cell lines with varying levels of expression. Since Fn14 was present on the cell surface, we initially assayed cellular adhesiveness to several immobilized extracellular matrix proteins. Cellular adhesion to the extracellular matrix is mediated primarily by the integrin family of heterodimeric transmembrane proteins (43, 44). Neither the vector control nor the three Fn14-HA-expressing cell lines adhered significantly above background levels when plated on collagen I, collagen IV, or laminin. This result is consistent with previous reports (45–47) and our results (data not shown) demonstrating that the α5 and α2 integrin subunits (which represent two of the major α-integrin subunits which bind collagen and/or laminin) are expressed at relatively low levels in NIH 3T3 fibroblasts. Both the control and the Fn14-HA-expressing cell lines could adhere to fibronectin or vitronectin, consistent with previous studies (45–48) and our results (data not shown) demonstrating that the αβ1 integrin subunits (which represent two of the major α-integrin subunits which bind collagen and/or laminin) are expressed at relatively low levels in NIH 3T3 fibroblasts. In comparison to the control V5 line, the two experimental cell lines expressing the highest Fn14-HA levels showed decreased attachment to fibronectin while all three Fn14-HA-expressing lines showed decreased attachment to vitronectin. In these assays, the level of Fn14-HA expression did not strictly correlate with the loss of cellular adhesiveness. This may reflect a saturation of the inhibitory effect at intermediate (for fibronectin binding) or low (for vitronectin binding) levels of ectopic expression. Additional experiments using only the control cell line and the experimental cell line expressing the highest level of Fn14-HA revealed that constitutive Fn14 expression inhibited cellular adhesion to various concentrations of immobilized vitronectin.

Nontransformed cells in culture are dependent on interactions with an adhesive surface for both cellular proliferation (49–52) and migration (42, 53, 54). Therefore, we performed serum-stimulated growth and chemotactic migration assays using the control cell line and the experimental cell line expressing the highest level of Fn14-HA. In these growth and migration experiments, it is likely that serum-derived vitronectin is the major attachment factor adsorbed to the tissue culture plastic or Transwell nuleopore membranes, respectively (55). We found that constitutive Fn14 expression inhibited both serum-stimulated NIH 3T3 cell growth and motility.

In conclusion, we have identified a growth factor- and tumor promoter-inducible immediate-early response gene on mouse chromosome 17, designated Fn14, which encodes a novel plasma membrane protein. Constitutive Fn14 expression in transfected NIH 3T3 cells reduces cellular adhesion to fibronectin and vitronectin and also inhibits serum-stimulated cell proliferation and migration. The molecular basis for these effects is presently under investigation, but we propose that Fn14 could be altering integrin subunit expression, ligand binding, or signal transduction. This could occur by direct binding of Fn14 to integrin subunits; alternatively, Fn14 could interact with other transmembrane proteins that mediate integrin function; for example, integrin-associated protein CD47 (56), calveolin (57), syndecan-2 or -4 (58, 59), or members of the tetraspanin family (60). Finally, it is possible that the transient expression of this protein in growth factor-stimulated cells may be important for the “de-adhesion” process associated with cell division and motility (61).
Mitogen Regulation of Fn14 Gene Expression

41. Albrecht-Buehler, G., and Lancaster, R. M. (1976) J. Cell Biol. 71, 370–382
42. Mitchison, T. J., and Cramer, L. P. (1996) Cell 84, 371–379
43. Hynes, R. O. (1992) Cell 69, 11–25
44. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) Annu. Rev. Cell Dev. Biol. 11, 549–599
45. Sanker, S., Mahooti-Brooks, N., Hu, G., and Madri, J. A. (1995) Am. J. Pathol. 147, 601–608
46. Defilippi, P., Olivo, C., Tarone, G., Mancini, P., Torrisi, M. R., and Eva, A. (1997) Oncogene 14, 1933–1943
47. Belkin, A. M., and Retta, S. F. (1998) J. Biol. Chem. 273, 15234–15240
48. Woodard, A. S., Garcia-Cardeña, G., Leong, M., Madri, J. A., Sessa, W. C., and Languino, L. R. (1998) J. Cell Sci. 111, 469–478
49. Folkman, J., and Moscona, A. (1978) Nature 273, 345–349
50. Dike, L. E., and Farmer, S. R. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 6792–6796
51. Renshaw, M. W., Ren, X.-Y., and Schwartz, M. A. (1997) EMBO J. 16, 5592–5599
52. Lin, T. H., Chen, Q., Howe, A., and Juliano, R. L. (1997) J. Biol. Chem. 272, 8849–8852
53. Lauffenburger, D. A., and Horwitz, A. F. (1996) Cell 84, 359–369
54. Palecek, S. P., Luftus, J. C., Ginsberg, M. H., Lauffenburger, D. A., and Horwitz, A. F. (1997) Nature 385, 537–540
55. Hayman, E. G., Pierschbacher, M. D., Ohtgren, Y., and Ruoslahti, E. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 4003–4007
56. Lindberg, F. P., Gresham, H. D., Schwarz, F., and Brown, E. J. (1993) J. Cell Biol. 123, 485–496
57. Wei, Y., Yang, X., Liu, Q., Wilkins, J. A., and Chapman, H. A. (1999) J. Cell Biol. 144, 1285–1294
58. Grané, F., Reina, M., Casaroli-Marano, R. P., Castel, S., Rocamora, N., Ureña, J. M., and Vilaro, S. (1999) Exp. Cell Res. 248, 439–456
59. Saucelis, S., Eckeltmayer, F., Denbez, F., Newlen, J. K., Mosher, D. F., Robinson, S. D., Hynes, R. O., and Goetinck, P. P. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 2805–2810
60. Maecker, H. T., Todd, S. C., and Levy, S. (1997) FASEB J. 11, 428–442
61. Greenwood, J. A., and Murphy-Ullrich, J. E. (1998) Microsc. Res. Tech. 43, 420–432