Abstract. We describe the construction in retroviral vectors and the expression of recombinant rat fibronectin (FN) cDNAs corresponding with the various alternatively spliced forms of FN. In NIH 3T3 cells, the exogenous rat FN subunits are efficiently secreted as heterodimers with endogenous mouse subunits. In contrast, in lymphoid WEHI231 cells, there is no endogenous FN synthesis and the recombinant FNs are secreted and can be purified as homogeneous proteins. We show that the purified recombinant FNs are biochemically and biologically functional. In basic assays for adhesion, spreading, cytoskeletal organization, and migration using various established adherent cell lines, different forms of FNs containing the different alternatively spliced segments show no marked differences in activity. We have used these recombinant FNs to investigate three systems in which earlier results had suggested potential differences between different forms of FN. First, all forms tested appear equally active in restoring normal morphology to a transformed cell line. Second, we detect minor differences in their ability to assemble into preexisting extracellular matrices. Finally, we report that only those forms of FN that contain the V segment will promote the spreading of a lymphoid cell line indicating that this segment confers additional biological functions for some cell types, a result that confirms and extends earlier data. These homogeneous, biologically active recombinant FNs will allow further studies of the role of the alternatively spliced segments of FN.

Fibronectins (FNs) are multifunctional extracellular matrix (ECM) glycoproteins that interact with other ECM components including fibrin, collagens, and heparin as well as with integrin receptors on cell surfaces. These interactions, probably collectively, promote vital cellular functions such as adhesion, spreading, cytoskeletal organization, cell migration, and matrix formation. These basic cellular properties in turn play important roles in a variety of biological processes, including cell migration during embryonic development, wound healing, and oncogenic transformation (Hynes and Yamada, 1982; Mosher, 1989; Hynes, 1986, 1990). In contrast with these complexities of function, FNs exhibit a relatively simple, modular structure (reviewed in Hynes, 1985, 1990; Mosher, 1989). The protein consists of a dimer of two subunits joined by disulfide bonds near their COOH termini. Each subunit is composed of a series of homologous repeating units of three types (types I, II, and III, see Fig. 1). Several binding activities of FN have been successively assigned to discrete parts of the molecules by using proteolytic fragments generated from plasma fibronectins (pFN). For example, the short peptide sequence Arg-Gly-Asp-Ser (RGDS) in the central region of pFN has been identified as a primary cell binding site (Pierschbacher and Ruoslahti, 1984a,b). More recently, however, by using fusion proteins containing various fragments of human pFN, Obara et al. (1988) discovered another site amino-terminal to the RGDS sequence that acts synergistically with it in promoting cell spreading. Furthermore, it has also been reported that the primary cell-binding domain and the heparin-binding domain of FN cooperate in functions such as cytoskeletal organization and neurite outgrowth (Woods et al., 1986; LeBaron et al., 1988; Rogers et al., 1987). Thus, the interactions of FNs with cells are complex, involving several different parts of the FN molecules.

Interestingly, three segments (designated in rat FN as EIIIB, EIIB, and V) in the central part of the molecule can be included or excluded because of alternative splicing of a single primary transcript (Kornbluth et al., 1985; Schwarzbauer et al., 1983, 1987a; Sekiguchi et al., 1986; Norton and Hynes, 1987; Gutman and Kornbluth, 1987; Zardi et al., 1987). The patterns of splicing are cell type specific; for example, hepatocytes synthesizing pFN exclude EIIB and EIIB, whereas fibroblasts and astrocytes synthesize FNs partially including these two segments (Kornbluth et al. 1984; Price and Hynes, 1985; Paul et al., 1986; Schwarzbauer et al., 1987a). Furthermore, recent experiments using in situ hybridization, nuclease protection and mAb techniques have demonstrated that the expression of various forms of FN is also regulated at different developmental stages, in a tissue-
and cell-specific fashion and in response to physiological and pathological processes (Vario et al., 1987; ffrench-Constant and Hynes, 1988, 1989; ffrench-Constant et al., 1989; Carnemolla et al., 1989). Together, these findings suggest the exciting possibility that alternatively spliced segments of FN may possess unique properties, therefore contributing to functionally distinct FN forms.

Humphries et al. (1986, 1987) have shown, using synthetic peptides, that the V segment (also called IIICS) contains cell-binding activities for certain cell types. To date, there are no indications of the functions of the EIIIA and EIIIB segments and it has been very difficult to carry out clean experiments testing the functions of any of these tissue-specific segments of FNs in the context of the intact molecules. All natural sources of FNs are mixtures of several different types of subunits which form many different dimeric forms of FN. Thus, the different dimeric forms contain more than one type of FN subunit and share the biochemical properties traditionally exploited for protein separation.

To study the structure-function relationships of rat FNs at a finer level and with particular interest in exploring the possible functions of the EIIIB, EIIIA, and V regions, we have recently undertaken a molecular genetic approach. A series of overlapping genomic clones covering the entire gene was isolated (Patel et al., 1987; Schwarzbauer et al., 1987a). cDNA clones corresponding to various parts and different forms of the FN mRNA were also isolated from a cDNA library or generated from genomic clones using a fusion rescue method (Patel et al., 1987; Schwarzbauer et al., 1987a, b). Parts of these cDNAs were expressed in eukaryotic cells using retroviral expression vectors (Schwarzbauer et al., 1987b). In this study, we first assembled full-length cDNA clones encoding eight different possible rat FN forms from available cDNA fragments. We then expressed these different rat FNs in heterologous mammalian cells that do not themselves produce endogenous FN. Pure homodimers of each rat FN form were then prepared from these expressing cell lines and subjected to various functional assays. We show here that recombinant FNs produced from heterologous mammalian cells are fully functional in promoting cell adhesion, spreading, migration, and cytoskeletal organization. We have compared the ability of the various forms to restore normal morphology to transformed cells and find them equivalent. Small differences were detected in their ability to assemble into preexisting matrices. Finally, only those forms of FN containing the V segment can promote spreading of a B lymphocyte cell line, consistent with a cell adhesion function for this alternatively spliced segment.

Materials and Methods

Cell Cultures

NIH 3T3 and ϕ2 cells were grown in DME plus 10% calf serum (CS, Gibco Laboratories, Grand Island, NY). Mouse B lymphocyte WEHI231 cells (Ralph, 1979) were kindly provided by D. Schatz (Whitehead Institute, Massachusetts Institute of Technology) and grown in RPMI 1640 medium plus 10% FCS (Gibco Laboratories). NRK, Rat1, and NIH3T3 cells were cultured in DME with 5% FCS. CHO cells were maintained in F-12 medium supplemented with 10% FCS. HUVEC cells were cultured in DME with 5% FCS. CHO cells were maintained in F-12 medium supplemented with 5% FCS. BHK cells were kindly provided by F. Grinnell (University of Texas, Dallas) and maintained in DME plus 10% FCS. Murine melanoma B16F10 cells were generous gifts of L. J. Fidler (M. D. Anderson Hospital, Houston) and cultured as described (Fidler, 1974).

Plasmid Construction

Retroviral vectors containing the 3' third of rat FN cDNA including or excluding EIIIA or V have been described previously (Schwarzbauer et al., 1987b). Overlapping cDNA clones covering the 5' regions of the gene were generated from the respective genomic clones, XR-FN-3, XR-FN-5, XR-FN-8, and Ar-FN-9 (Patel et al., 1987; Schwarzbauer et al., 1987a), using a fusion rescue method as outlined before (Schwarzbauer et al., 1987a, b). These cDNA clones, including or excluding EIIIB, were recombined, using unique restriction sites, with the existing 3' cDNAs to give rise to full length cDNAs encoding rat FN. All possible combinations of EIIIB, EIIIA, and V were made and the constructs confirmed by restriction mapping. These full-length cDNAs extend from a Bal I site (TGCGCA) 50 nucleotides upstream of the initiator codon of rat FN (Patel et al., 1987) to a Sac II site (CCGCGG) in the 3' untranslated region (Schwarzbauer et al., 1983, 1987b; Patel et al., 1987). They include the entire coding region of the various forms of rat FN and the 3' untranslated region included in the earlier retroviral constructs (Schwarzbauer et al., 1987b). Thus, these constructs include the natural signal and propeptide segments of rat FN to allow normal secretion and processing. The full length clones were inserted into the retroviral expression vector pLJ to generate pLJ-FN plasmids. pLJ is a derivative of pDOL (Korman et al., 1987; Schwarzbauer et al., 1987b), from which the 5' splice site has been completely removed, and was generated and provided by J. Schwarzbauer. cDNAs to be expressed are inserted at a cloning site downstream of the 5' MLV LTR. After the cloning site is an SV-40 origin/promoter/enhancer segment and the neomycin resistance (neo) gene driven by the SV-40 early promoter and a pBR322 origin of replication.

Transfection of ϕ2 Cells and Infection of NIH 3T3 and WEHI231 Cells

Establishment of virus-producing ϕ2 cells and infection of NIH 3T3 and WEHI231 cells were performed as previously described (Schwarzbauer et al., 1987b; Landau et al., 1987) with the following modifications. After infection, NIH 3T3 cells were selected for neo expression in G418 (Gibco Laboratories) at a concentration of 0.5 mg/ml. A subset of G418-resistant clones was then isolated and clones were expanded for further analysis. Infected WEHI231 cells were selected with G418 at a concentration of 3 mg/ml. The selected pool of cells was then cloned by limiting dilution. Single cell clones that produced the highest amounts of recombinant FNs, as determined by immunoprecipitation, were expanded for further analysis.

Northern Blot Analysis

Total RNA was isolated from NIH 3T3 cell clones by guanidinium thiocyanate extraction followed by centrifugation through CsCl as described by Chirgwin et al. (1979). 20 μg of RNA were electrophoresed in 0.8% agarose gels containing 1.1 M formaldehyde as described (Lehrach et al., 1977). After electrophoresis, gels were stained with ethidium bromide to visualize the position of 28S-18S ribosomal RNA and the relative RNA content in each lane. The RNA was then transferred to Zeta-Probe blotting membranes (Bio-Rad Laboratories, Richmond, CA), which were processed using protocols recommended by the manufacturer. Duplicate RNA samples were used for hybridizations with probes corresponding to rat FN cDNA or the neo gene. The probes were labeled with 32P or 35S by random priming (Feinberg and Vogelstein, 1984).

Metabolic Labeling, Immunoprecipitation, Gelatin and Heparin Binding Assays, and SDS-PAGE

Cells were labeled for 20–24 h with media containing a reduced amount of unlabeled methionine (10% of that in normal media) and 25 μCi/ml [35S]methionine (Tran35S-label, ICN Radiochemicals, Irvine, CA). Conditioned media were immunoprecipitated using either a rabbit anti-rat FN serum R61 and goat anti-rabbit IgG or a mouse monoclonal anti-rat FN M9 (a generous gift of M. Chiquet, Carnegie Institute, Baltimore, MD) and goat anti-mouse IgG, as described (Choi and Hynes, 1979). Immunoprecipitates were analyzed either with or without reduction by electrophoresis through SDS-PAGE followed by fluorography. Direct binding of FNs in the conditioned media to gelatin-coupled Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was carried out as described (Price and Hynes, 1985). The bound materials were also eluted by 4 M urea in PBS and used directly for binding assays with heparin-coupled Sepharose (Pharmacia Fine Chemicals) as described by Price and Hynes (1985).
Purification of Recombinant Rat FNs

Recombinant FNs produced from expresser WEHI231 cell clones were purified by affinity-chromatography using a gelatin-coupled Sepharose column as described by Engvall and Ruoslahti (1977). Briefly, the expressing clones were grown to saturation in 3 liters of growth medium. The cells were then washed with PBS and resuspended in 10 liters of RPMI1640 plus 5% FCS that had been passed through gelatin-Sepharose 4B to deplete FN in the serum. The cells were incubated further for 3 d and the conditioned media were concentrated and subsequently loaded onto a gelatin-Sepharose 4B column. Recombinant FNs were eluted using 4 M urea in CAPS buffer (10 mM cyclohexylamino propane sulfonic acid (CAPS), 150 mM NaCl, 2 mM EDTA, 2 mM PMSF, pH 11). The peak fractions as determined by UV absorption at 280 nm were pooled and dialyzed against CAPS buffer to remove urea. The final concentrations were determined again by UV absorption and also confirmed by comparison with protein standards on SDS-PAGE followed by Coomassie blue staining. Typical yields were 3-5 mg of purified recombinant FN from 10 liters of culture supernatant.

Cell Spreading Assay

The biological activities of recombinant FNs were determined by a quantitative cell spreading assay modified from that described by Yamada and Kennedy (1984) and Obara et al. (1988). Restricted areas of tissue culture plates were incubated with 25 μl of serial dilutions of recombinant FNs for 2 h at room temperature, followed by incubation with 2 mg/ml heat-treated (10 min at 80°C) BSA in PBS for 2 h at 37°C and extensive washing with PBS. Adherent cell lines were harvested with brief trypsinization and then washed with PBS containing 0.5 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO). The cells were added to coated plates at 10^5/ml in growth medium without serum. Suspension WEHI231 cells were washed with PBS and added at 2 × 10^6/ml. After 2 h of incubation at 37°C, the plates were washed with PBS and fixed in 3.7% formaldehyde in PBS. Percent cell spreading was then determined by counting three random fields (200-300 cells) using a Nikon inverted phase-contrast microscope.

Other FN Functional Assays

The effect of various recombinant FNs on NIH3T3 cells was examined as described by Ali et al. (1977). 2 ml of growth medium containing 2 × 10^4 cells were seeded in 35-mm dishes. After 48 h, FNs were added in 100 μl PBS to give the desired concentration. Photographs were taken on the Nikon inverted phase-contrast microscope 24 h later.

To examine their effects on cytoskeletal organization, recombinant FNs were used to coat coverslips at various concentrations. Murine melanoma B16F10 cells or fibroblastic BHK cells were then plated onto the coated coverslips in the absence of serum. 2 h later, the distribution of actin bundles and vinculin was visualized by immunofluorescence. Briefly, cells were rinsed twice in PBS and fixed for 15 min in a freshly prepared 4% solution of paraformaldehyde (Fluka Chemical Co., Bern, Switzerland) in PBS, rinsed and permeabilized with 0.5% NP-40 in PBS for 15 min. Cells were stained with mouse mAb against vinculin (Sigma Chemical Co.) in 10% normal goat serum in PBS for 30 min at 37°C. After three washes with PBS, the second antibody mixture (rhodamine-conjugated goat anti-mouse IgG and fluorescein-conjugated phalloidin in 10% normal goat serum in PBS, Cappel Laboratories, Cochranville, PA) was added and incubated for 30 min at 37°C. After three washes, coverslips were mounted in Gelvatol and examined using a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) and photographed using Kodak Tri-X film (Eastman Kodak Co., Rochester, NY).

Cell migration assay was carried out using a Micro Chemotaxis Chamber (Neuro Probe Inc., Cabin John, MD) as described by McCarthy and Furcht (1984). Incorporation of recombinant rat FNs into the extracellular matrix of NIH 3T3 cells was determined by indirect immunofluorescence as described above except that mouse monoclonal M9 and rabbit antiserum R61 were used as the primary antibody, and fluorescein-conjugated goat anti-
mouse IgG and rhodamine-conjugated goat anti-rabbit IgG were used as the second antibody.

Results

Preparation of Retroviral Expression Vectors Encoding All Eight Different Rat Fibronectin Forms

The structure of rat FNs composed of three types of repeating peptide units termed Type I, II, and III is diagrammed in Fig. 1. Various parts of the molecules that interact with fibrin, collagen, heparin, and cell surfaces are illustrated above the diagram. Three regions (EIIIA, EIIIB, and V) that can be included or excluded in different FN forms due to alternative splicing are also highlighted. These different rat FN forms will be abbreviated as O, B, A, V, BV, AV, BA, and BAV forms to indicate the variably included regions which they contain.

Full length cDNA clones encoding eight possible rat FN forms were assembled from available cDNA fragments as described in Materials and Methods. The FN coding sequences in these constructs are identical with the native ones including the signal and pro segments of FN. The full length clones were then inserted into the retroviral expression vector pLJ to generate pLJ-FN plasmids. These plasmids were transfected into 293 cells to prepare recombinant retroviruses containing FN genes as detailed in Materials and Methods. The recombinant viruses were used to infect murine recipient cells including fibroblastic NIH 3T3 and lymphoid WEHI231 cells. The neomycin-resistance gene (neo') encoded in the vector under the control of the SV40 early promoter allowed selection (with G418) of infected clones. These clones were subsequently analyzed for FN expression driven by the viral 5'LTR promoter.

Stable Expression of Rat Fibronectin in NIH 3T3 Cells

It was necessary first to test the function of these recombinant FN genes to ensure appropriate and efficient transcription, translation, and posttranslational processing. To examine the expression of recombinant rat FNs in the context of a cell type which normally produces, secretes, and assembles FN, multiple G418-resistant clones were isolated from infected murine NIH 3T3 cells. Total cellular RNA was prepared from these clones as well as from parental 3T3 cells and then subjected to Northern blot analysis. Fig. 2 shows results from one such experiment examining the expression of the BAV form of FN. A shows that two transcripts were detected from three individual clones infected with retrovirus containing the BAV form FN gene when the neo' gene was used as probe. The 2.456- and 3.1-kb messages correspond with the sizes expected for the full-length genomic transcript from the viral LTR and subgenomic RNA from the SV-40 promoter as diagrammed in C. As expected, no band was detected in RNA isolated from parental 3T3 cells (lane -). RNA

Figure 1. Rat FN structure and binding sites. The rat FN subunit composed of type I, II, and III repeating units (boxes) is shown schematically. Various domains that interact with fibrin, collagen, heparin and cell surfaces are indicated above. The EIIIB and EIIIA segments are shown with the filled squares while the V region is represented by a shaded box.
Figure 2. Northern blots. RNA was isolated from three NIH 3T3 cell clones expressing the BAV form of rat FN as well as control parental cells or cells infected with virus containing the vector pLJ alone, as indicated. Duplicate samples were electrophoresed, transferred and hybridized with probes corresponding to neo' gene (top left) or rat FN cDNA (top right) as described in Materials and Methods. The positions of mRNAs corresponding to rat FN (rFN), endogenous mouse FN (mFN), and neoresistance gene (Neo-R) are marked in the center. The expected genomic and subgenomic transcripts from pLJ-FN plasmids are diagrammed below (bottom). The viral LTR promoters are indicated at both ends while FN cDNA, neo-R gene, and SV-40 promoter are represented by boxes filled with wider lines, thinner lines, and dots respectively.

isolated from cells containing pLJ vector alone included the 3.1-kb subgenomic RNA and a minor band migrating at 3.9 kb, which corresponds with a transcript derived from the 5' viral LTR. The identity of the 11.6-kb transcript was confirmed by hybridizing the same blot with a probe derived from rat FN cDNA (B). This probe also detects the endogenous mouse FN message (8.1 kb), which is present in rat FN expressor clones as well as in the control cell lines. The rat FN mRNA signal was ~10% of the signal for endogenous mouse FN mRNA. Therefore, the recombinant rat FN genes appear to function exactly as expected. However, because interspecies FN heterodimers form in these cells, it is not possible to assay the behavior of pure recombinant FNs.

To detect and quantitate secretion of recombinant rat FNs from 3T3 cells, conditioned supernatants harvested from [35S]methionine-labeled cells were carried out with a mouse mAb, M9, specific for rat FN or with an antirat FN polyclonal serum R61. SDS-PAGE analysis of immunoprecipitates from representative clones expressing O, B, V, or BV rat FN forms is shown in Fig. 3. As shown in the left panel, all these forms of rat FN were secreted into the medium as proteins with molecular masses around 220–250 kD. The slight differences in molecular weight are as expected from their differences in polypeptide chain by including neither, either or both of the EIIIB and V regions. As seen most evidently in cells secreting the O form of rat FN, the endogenous mouse FN (a mixture of different forms but mostly larger than the O form) is coprecipitated with M9. This result shows that the recombinant rat FN can form dimers with the endogenous mouse FN which has been observed before for 3T3 cells expressing the COOH-terminal third of rat FNs (Schwarzbauer et al., 1987b). The two leftmost lanes show that no endogenous FNs were precipitated by M9 from supernatants of 3T3 parental cells or clones infected with vector alone. The right panel shows the immunoprecipitations with the polyclonal anti-FN serum, demonstrating that all these clones secrete comparable amounts of total fibronectins. Estimates from densitometry of the autoradiographs showed that the recombinant rat FNs secreted from 3T3 cell clones represent ~10% of the total FNs produced by these cells. This estimate corresponds well with the 10% rat FN transcripts compared with total fibronectin message as determined from Northern blot analysis (Fig. 2). Thus, the chimeric rat FN mRNAs are efficiently translated and the rat fibronectins expressed in stable infected NIH3T3 cell clones are efficiently processed, assembled into dimers and secreted into the medium. These cells also assemble the recombinant rat FNs into extracellular matrix (data not shown but see below).

Therefore, the recombinant FN genes appear to function exactly as expected. However, because interspecies FN heterodimers form in these cells, it is not possible to assay the behavior of pure recombinant FNs.

Isolation of B Lymphoma WEHI231 Cell Clones Producing Recombinant Fibronectins

To prepare large quantities of homogeneous recombinant FNs for further studies, recombinant viruses containing genes for O, B, A, and V forms of rat FNs were used to infect a murine B lymphoma cell line WEHI231 which does not produce any endogenous FN (as shown below). WEHI231 cells were infected by coculture with virus-producing V2 cell
Figure 4. Analysis of recombinant FNs produced by WEHI231 cells. Conditioned culture media from [35S]methionine-labeled cells were each divided into four aliquots and subjected to the following treatments. The first two aliquots were immunoprecipitated with rabbit antiserum R61 and electrophoresed either under nonreducing (A) or reducing (B) conditions. The positions of the FN monomer and dimers (FN2) are marked at the left. The third aliquot was incubated with gelatin-Sepharose, washed and eluted with electrophoresis sample buffer followed by SDS-PAGE (C). The last aliquot was incubated with gelatin-Sepharose and the bound materials were eluted with urea in PBS and then incubated with heparin-Sepharose. The bound proteins were then dissolved in sample buffer and analyzed by SDS-PAGE (D). The molecular weight markers shown on the right are 200, 97, and 68 kDa.

Figure 5. Purification of recombinant FNs. Recombinant rat FNs were purified by gelatin-Sepharose affinity chromatography as detailed in Materials and Methods. Aliquots of the purified proteins were subjected to SDS-PAGE (two lanes for each form) and visualized by Coomassie blue staining. The molecular mass marker of 200 kDa is shown on the right.

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The spreading of B16F10 melanoma cells on the A form of FN. B16F10 melanoma cells were seeded on tissue culture plates coated with various amounts of the A form of FN as indicated. 2 h later, cells were rinsed and fixed with formaldehyde in PBS. Photographs were taken on a Nikon inverted phase-contrast microscope. Similar results were observed with the other forms of FN.

Figure 6. The spreading of B16F10 melanoma cells on the A form of FN. B16F10 melanoma cells were seeded on tissue culture plates coated with various amounts of the A form of FN as indicated. 2 h later, cells were rinsed and fixed with formaldehyde in PBS. Photographs were taken on a Nikon inverted phase-contrast microscope. Similar results were observed with the other forms of FN.

purified FNs are slightly different, in agreement with differences in their primary sequences. These results also indicated that recombinant FNs produced by WEHI231 lymphocytes are indeed homodimers free of contamination with FN from natural sources.

The biological activities of recombinant FNs were first tested using a cell spreading assay modified from that described by Yamada and his colleagues (Yamada and Kennedy, 1984; Obara et al., 1988; see Materials and Methods for details). Several different adherent cell lines, including NIH
Figure 7. B16F10 melanoma cells spread on all forms of recombinant FNs. The spreading of B16F10 melanoma cells on all four forms of recombinant FNs were determined and quantitated as described in Materials and Methods. The average scores from three independent experiments are plotted here. The SDs were <10% on all points and are not shown.

3T3, NRK, Rat1, CHO, BHK, and murine melanoma B16F10, were tested in these assays. We found that all four forms of recombinant FNs (O, B, A, and V) promote adhesion and spreading of all the cell lines tested here. Fig. 6 shows one representative experiment with the mouse melanoma cell line B16F10. On control substrata coated only with BSA, the cells either did not attach, or spread poorly (top). On substrata coated with a low amount of recombinant FN (in this case 2 µg/ml A form), significantly increased numbers of cells attach, but relatively few assume a well-spread morphology (center). When plated on higher doses of FNs (16 µg/ml A form), the majority of the cells adhere and spread well (bottom). These results, as well as similar experiments with the other cell lines mentioned above, indicated that all four forms of FNs tested are biologically active in promoting cell adhesion and spreading. Furthermore, it appeared that all these forms of recombinant FNs promote cell adhesion and spreading to a similar extent. This observation was confirmed by quantitative measurements shown in Fig. 7 for B16F10 melanoma cells. The percentages of cells spreading are plotted as average scores for three independent experiments and the SDs were <10%. This experiment demonstrated that these four forms of recombinant FNs have similar dose-response curves in promoting B16F10 cell spreading, reaching saturating concentration at ~10 µg/ml. This is comparable with the doses of pFN required to produce similar effects (data not shown). These results suggest that, in the basic adhesion and spreading assays with these established adherent cell lines, all forms of FN tested are equivalent (see Discussion).

Other Biological Properties of Recombinant Fibronectins

Having established that the recombinant FNs were func-
Cytoskeletal organization of B16F10 melanoma cells spread on recombinant FNs. B16F10 melanoma cells were plated on coverslips coated with various forms of recombinant FNs at 20 μg/ml and their cytoskeletal organization was depicted by immunofluorescent staining of actin bundles (left) and vinculin (right). Note that all three forms (O, V, and B) promoted cytoskeletal organization of these cells. Similar results were obtained with the A form.

Through their transmembrane integrin receptors, extracellular FNs can induce cytoskeletal organization including organized actin bundles and focal contact formation. To examine these transmembrane effects, B16F10 melanoma cells were cultured on substrata coated with various recombinant FNs. 2 h later, cells were fixed and stained for F actin and vinculin distribution using double-label immunofluorescence. When low concentrations of the various FNs were used, actin bundles were visualized in only a small percentage of cells while diffuse and unorganized patterns were evident for most cells. Similarly, few focal contacts were detected as determined by staining for vinculin (top, Fig. 8). When plated on higher concentrations of FNs however, extensive microfilament bundles were detected for the majority of cells and discrete focal contacts were localized at termini of actin bundles (bottom, Fig. 8). These results indicated that recombinant FN V form was able to induce cytoskeletal organization. Similar effects were observed for the other forms of FNs obtained from WEHI231 cells (O, V, B forms are shown in Fig. 9).

Next, we examined the ability of recombinant FNs to promote cell migration. A modified Boyden chamber assay as described by McCarthy and Furcht (1984) was employed. Several cell lines including NIH3T3, BHK, B16F10, and Nii8.HSV were tested in these assays. Fig. 10 shows a representative result with Nii8.HSV cells in response to A form FN. In control wells with no FN added, very few cells migrated through the membrane with holes of suitable size (left). When FN was added to the other side of a membrane, significant numbers of cells migrated through membranes (right). Cells per unit area were counted; all four forms of FNs promoted cell migration to similar extents (Table I).

Therefore, as for adhesion and spreading, the four forms of FNs have similar basic binding functions of FN in cell adhesion and spreading, we sought next to investigate their efficacy in more complex biological functions.

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Figure 10. Recombinant FN-mediated migration of Nil8-HSV cells. Cell migration assay was carried out as described in Materials and Methods. Cells that migrated through the membrane in response to FN were visualized by Giemsa stain. (Left) shows the control where very few cells migrated when no FN was added. (Right) shows cells migrated when the A form of FN was added to the buffer. Similar results were obtained with the other forms of FN.

of FN tested (O, B, A, and V) all appeared equally effective in promoting cytoskeletal organization and cell migration in the cell types tested.

We turned next to investigate the functions of the different forms in several assays where there have been suggestions that different forms of FN might differ in effectiveness. One striking morphological phenotype of transformed cells is their reduced adhesion to substrata. At least in some cases, the reduction in adhesion can be accounted for by their reduced levels of cell surface-associated FNs (Yamada et al., 1976; Ali et al., 1977; Willingham et al., 1977). Furthermore, addition of exogenous FNs to these transformed cells increases their attachment to the substrata, and causes them to spread out and adopt a more flattened morphology resembling that of normal cells. Yamada and Kennedy (1979) reported that cellular FNs presumably containing some

Table I. Migration of Nil8-HSV Cells in Response to Fibronectin

|        | 1.25 μg/ml | 2.5 μg/ml | 5.0 μg/ml | 10.0 μg/ml |
|--------|------------|-----------|-----------|------------|
| O      | 38.7 ± 6.1 | 138.0 ± 4.0| 224.3 ± 17.5| 211.7 ± 12.1|
| B      | 35.3 ± 4.2 | 153.3 ± 3.8| 211.3 ± 19.5| 216.3 ± 16.0|
| A      | 43.3 ± 6.4 | 162.7 ± 21.2| 206.0 ± 5.3 | 215.3 ± 12.5|
| V      | 42.7 ± 5.9 | 159.0 ± 10.8| 185.0 ± 4.4 | 222.7 ± 18.6|

Cell migration assays were carried out as described in Materials and Methods. The number of migrated cells per high-power field (x250) was counted and the average score of three independent experiments is shown with SDs. In the absence of FN, fewer than five cells migrated.

EMB and EMA segments were 50-fold more effective than pFNs lacking these two segments in reverting the morphology of transformed cells. Others, however, did not detect the same differences between plasma and cellular FNs (Hynes et al., 1978). Using purified homodimers of recombinant FNs, we carried out similar experiments on HSV-transformed hamster Nil8 cells to determine the contributions of EIIIB and EIIIA in this activity. As shown in Fig. 11 (top), a substantial population of Nil8.HSV cells are rounded and detached from the substrate. The middle and bottom panels show that cells assumed a more flattened and aligned morphology upon addition of the recombinant FN A form. Similar effects were also observed for O, B, V forms of FNs (not shown). The differences in dose response among the different forms were, at most, two to threefold in different experiments. Therefore, in agreement with results obtained from basic adhesion and spreading assays using adherent cells, the ability of FN to revert the morphology of these transformed cells does not appear to reside in the EIIIB, EIIIA, or V regions.

Although pFN can incorporate into preexisting extracellular matrices in vitro (Hayman and Ruoslahti, 1979) and in vivo (Oh et al., 1981), this form of FN is generally a soluble protein. Purified FN from cells is generally of lower solubility (e.g., Yamada et al., 1977; Alexander et al., 1978, 1979) and it has often been proposed that it could be more effective in assembly into extracellular matrices. Accordingly, we compared the ability of recombinant FNs lacking (O and V forms) or containing (A and B forms) the segments charac-
Figure 11. Effects of the A form of FN on the morphology of Nil.8-HSV. Various amounts of the A form of recombinant FN were added to subconfluent Nil.8-HSV cells as described in Materials and Methods. 24 h later, pictures were taken under the phase contrast microscope. Note the flattened morphology of cells treated with recombinant FN (middle and bottom) in comparison with the control (top). Similar results were obtained with the other forms of FN.
Incorporation of recombinant FNs into the existing matrix of NIH3T3 cells. Exogenous recombinant FNs at indicated concentrations were added to confluent NIH3T3 cells and incubated for 20 h. The incorporation of the recombinant rat FNs into the matrix was visualized by immunofluorescence using a rat-specific monoclonal M9 (right) or a rabbit polyclonal R61 (left).

...rated into the existing matrices. However, we noted two- to threefold differences in the doses of recombinant FNs required to give a particular level of rat FN-specific fluorescence. The O and V forms required higher levels added than did the A and B forms to give equivalent staining. Minimum doses for detectable M9 staining were 10 μg/ml for B or BAV and 30 μg/ml for V. Fig. 12 shows approximately equivalent incorporation of rat FN at 30 μg/ml B form and 90 μg/ml V form. These results suggest that inclusion of the EIIIA or EIIIB segments characteristic of tissue FN may enhance the ability of FN to incorporate into existing matrix.

A Unique Function of Alternatively Spliced V Segment in Promoting Lymphocyte Spreading

Our observation that O, B, A, and V FNs promote adhesion and spreading of adherent cell lines to a similar extent may not be surprising because all these cell lines contain integrin αβ₁ on their surfaces (our unpublished data). It is known that αβ₁ is the predominant FN receptor that interacts with the RGDS sequence in FN (Pytel et al., 1985; Ruoslahti and Pierschbacher, 1987; Buck and Horwitz, 1987; Hynes, 1987). The RGDS sequence is included in all four forms of FNs tested, thus, it is both important and necessary to test other cell lines, preferably ones lacking αβ₁, to detect any possible functional differences in cell adhesion among these forms.

Earlier papers had suggested that some lymphoid cells might bind to a site in FN distinct from the RGDS sequence (Bernardi et al., 1987; Liao et al., 1989). This site was mapped to the COOH terminal part of FN, in or near the heparin-binding site. Since we were expressing the recombinant FNs in a lymphoid cell line, WEHI231, we inves...
Figure 13. WEHI231 cells spread only on the V form of FN. WEHI231 cells were seeded on surfaces coated with the A, B, O, or V forms of FNs (A, B, C, and D, respectively). 2 h later, cells were rinsed and photographed in a phase-contrast microscope. Note that only cells on the V form of FN assumed a well-spread morphology with clearly visible nuclei.

We investigated the response of these cells to various forms of FN. When the expression pattern of the integrin β1 subfamily was examined for mouse WEHI231 cells, we found on the surface very little or no αvβ1 or αvβ3, two integrin complexes known to interact with FNs (data not shown). However, we noticed that a proportion of WEHI231 cells expressing V FN were attached and even spread on the bottom of culture flasks, whereas those expressing the O, B, or A forms and parental WEHI231 cells all grew as suspension cultures. However, these expressor cell lines were isolated single-cell clones and they produce different amounts of FNs. Thus, their different behavior could be either a clonal difference or caused by differences in the amount of FNs accumulated in the media rather than by specific effects of the V segment.

To clarify this issue, purified recombinant FNs were used in experiments analogous to those described for adherent cells. It has been observed before that WEHI231 cells can attach to pFN although their nonspecific attachment to BSA is quite high (Bernardi et al., 1987). We also observed that WEHI231 cells can partially attach to BSA-coated substrata. Nevertheless, our assays measuring percent of spreading cells avoided this background problem since none of the cells spread on BSA-coated surfaces. Figure 13 shows a representative experiment where the A, B, O, or V recombinant FNs were used. It is evident that none of the A, B, or O FN forms promote lymphocyte spreading (A, B, and C), whereas V FN did so significantly (up to 70%, D). These results therefore indicated that the alternatively spliced V segment functions to promote spreading of B lymphocytes in vitro, presumably via some other cell surface receptor (see Discussion).

Discussion

In nature, FNs are usually mixtures of several distinct forms resulting from the alternative splicing at three regions of the gene transcript (EIIIB, EIIIA, and V in rat, Patel et al., 1987; Schwarzbauser et al., 1987a). The differential expression of various forms is both developmentally regulated and tissue
and cell specific, suggesting that there may be functional significance to the heterogeneity (Varti et al., 1987; ffrench-
Constant and Hynes, 1988, 1989; Carnemolla et al., 1989). For instance the EIIIA and EIIIB segments are prevalent
when there is abundant cell proliferation and migration, either during development (ffrench-Constant and Hynes, 1988), or during wound healing (ffrench-Constant et al., 1989) and the EIIIA segment is selectively excluded by cells such as hepatocytes and chondrocytes that do not assemble FN-rich matrices (ffrench-Constant and Hynes, 1989). These descriptive studies allow one to develop hypotheses for the potential roles of the alternatively spliced segments but it is necessary also to test such hypotheses. This has been difficult because the form of FN available in quantity is pFN, which lacks EIIIA and EIIIB, and FN from other sources, even when available, is a mixture of various forms. The recombinant FNs we describe here offer a solution to this difficulty. By expressing these recombinant FNs in a cell type which expresses no endogenous FN we are able to obtain pure homogeneous FNs with any desired combination of alternatively spliced segments.

In the present report, we concentrated on four forms of rat FNs containing none or one of the extra segments (called O, B, A, and V forms, respectively). We found that all these four forms were expressed efficiently in both fibroblastic NIH 3T3 cells and lymphoid WEHI231 cells via retroviral expression vectors. The recombinant FNs can assemble into dimers and be secreted into the media. They bind to gelatin and heparin efficiently and specifically in vitro. Furthermore, we showed that recombinant FNs produced by WEHI231 cells were fully functional in a variety of biological assays including cell adhesion, spreading, migration, cytoskeletal organization, and matrix formation.

Although all four forms of FNs tested can form homodimers to some degree, the V form of FN did so most effectively. This is in agreement with previous observations by Schwarzbauer et al. (1987b, 1989) who reported that the V segment is necessary for dimer formation in NIH3T3 cells and showed that pFN is predominantly a heterodimer of V and O subunits. However, in our system, ~50% of the O form of FN is dimeric, indicating that the V segment is not absolutely required for dimer formation in WEHI231 cells. Interestingly, inclusion of the EIIIA or EIIIB segment appeared to reduce dimer formation (Fig. 4). It is unclear how the monomers arise and are secreted. The two cysteine residues normally involved in intersubunit disulfide bonds may be free or form atypical disulfide bonds. The significance of these results is not clear since virtually all endogenous FN secreted by cells is dimeric. Most cellular FNs which contain EIIIA or EIIIB also contain V. It remains to be investigated how efficiently dimers form from recombinant FNs containing two or three extra segments. Perhaps there is a requirement for heterodimerization which in pFN is satisfied by the presence of V and V (i.e., O) forms and in other forms of FN is satisfied by the presence of mixtures of A and B forms. In spite of their differences in dimerization, all four forms of FNs seemed to be secreted efficiently into the media. Thus, dimer formation is not necessary for extracellular transport of FNs. Furthermore, their abilities to interact with collagens and heparin were not affected in vitro, which is not surprising since these biochemical activities can be retained even by monomeric proteolytic fragments (Mosher, 1989; Hynes, 1990).

Turning to the biological properties of the various recombinant FNs, one can note several things. First, all appear equally functional in the basic assays of cell adhesion, spreading, migration, cytoskeletal organization, and matrix assembly, at least when assayed with a variety of established cell lines. This again is not particularly surprising because pFN is active in these assays as are various proteolytic fragments of pFN and cellular FN. The various forms of FN share the great majority of the segments including the well-defined domains for binding to cells, heparin, and collagen (Fig. 1). It is to be expected that they will share many functions as we report here. However, note that the cells tested here are all established cell lines, which we used for purposes of comparison with the previous literature on FN functions. These experiments establish that the recombinant FNs behave as expected and prepare the ground for analyses with other cell types and in more complex biological assays.

In initial extensions of these experiments, we have reexamined three questions arising from the earlier literature concerning potential differences among different forms of FN. The first of these concerns the ability of FNs to revert the morphology of some oncogenically transformed cells toward normal (Yamada et al., 1976; Ali et al., 1977). Yamada and Kennedy (1979) reported that FN purified from plasma (cFN) was 50-fold more effective in this assay than was FN purified from plasma (pFN) and suggested that this was a functional difference between pFN and cFN. To test whether this reported difference in function is dependent on the presence of any of the alternatively spliced segments, we tested homogeneous recombinant FN forms containing each or none of the three segments. We were unable to detect significant differences in activity among the four forms tested; at most, they differed by two to threefold in efficacy in this assay. It remains possible that differences will emerge when recombinant FN forms are tested which contain more than one alternatively spliced segment. At present, it appears possible that the reported differences between cFN and pFN in this assay are not due to alternative splicing of FN and may instead arise from differences in preparation or minor contaminants.

The second difference in function of pFN and cFN which has been suggested in the literature is in their ability to assemble into matrices. Although pFN can assemble into matrices both in vitro (Hayman and Ruoslahti, 1979) and in vivo (Oh et al., 1981), the reported differences in solubility between the two forms (Yamada et al., 1977; Alexander et al., 1978, 1979) have been adduced to suggest that pFN, which is normally a soluble protein in plasma, may be less effective in forming matrices than the forms of FN produced by cells and found in tissues. To test whether the inclusion of any of the alternatively spliced segments enhances the ability of FN to assemble into preexisting matrices, we tested various forms of recombinant FN. As described in the Results section, those forms which contain the EIIIB or EIIIA segments did appear to be more efficient (Fig. 12). Further work will be necessary to investigate this possibility in experimental cell systems that do not contain endogenous FN. The WEHI231 producer cells are incompetent in assembly of extracellular matrix but the retroviral constructs described
here can be used to insert rFN genes into a wide variety of cell types so it should be feasible to test the ability of FN forms with one, two, or three of the alternatively spliced segments to construct a matrix de novo.

The one major difference which we did detect among the different forms of FN was in promoting the spreading of WEHI231 lymphoid cells. In this assay, only the V form of FN was active (Fig. 13). Humphries et al. (1986, 1987) have shown that a synthetic peptide corresponding to part of the human FN V region (also called IIIICS) can promote adhesion and spreading of mouse melanoma B16F10 cells. However, we did not detect significant differences in the ability of the O and V forms of recombinant rat FN to promote B16F10 adhesion and spreading. Although we can not exclude completely the possibility that material and experimen-tal differences are responsible for our different observations, we favor instead the following explanation. B16F10 melanoma cells may possess two receptor systems that interact with FNs, perhaps on different sites. It is likely that these two sites reside in the classic RGDS sequence with its synergy site and in the CSI (V25) site identified by Humphries et al. (1986, 1987). Both sites can mediate the adhesion and spreading of B16F10 cells to some degree. However in the whole molecule (such as our O and V forms), the effect of the V region site may be masked either due to conformational constraints or due to a relatively lower efficiency compared with the RGDS site under our assay conditions. In other words, that we did not find that the V form is twice as effective as the O form suggests that the V region site is not equally active as the RGDS sequence. However, the limits of sensitivity of our assay do not allow us to conclude that the V25 site is totally inactive in our experiments. In any case, it appeared that the RGDS site was the predominant cell-interacting domain for B16F10 melanoma cells in our in vitro assays. In agreement with this, we found abundant expression of $\alpha_6\beta_1$ integrin on the surface of these cells (our unpublished results). This integrin is the primary receptor for the RGDS site in FN (Pytela et al., 1985; Ruoslahti and Pierschbacher, 1987; Buck and Horwitz, 1987; Hynes, 1987). It seems likely that, in vivo, both of these two adhesion systems may function and either one can be dominant in certain situations.

This example of the $\alpha_6\beta_1$/RGDS system obscuring other cell interacting sites makes clear the desirability of using cell lines lacking $\alpha_6\beta_1$ integrin to detect any possible functional differences in promoting cell adhesion and spreading among these forms of recombinant FNs. In the present study, we found that the B lymphoid cell line WEHI231, which lacks $\alpha_6\beta_1$, spread only on the V form of FN, but not on the other forms. One trivial explanation for this would be that the V form of FN exists mostly as dimers and only dimeric FN can interact with WEHI231 cells. This is unlikely because the O form (with 50% as dimers) did not promote WEHI231 cell spreading at all. These results therefore suggest a unique function for the alternatively spliced V segment in promoting lymphocyte spreading. Bernardi et al. (1987) and Liao et al. (1989) have recently shown that FN can increase adhesion of several B lymphoid cell lines including WEHI231 cells. These workers have localized a site near the high-affinity heparin-binding domain of FN which is active in increasing adhesion of several B cell lines. Since the V segment is close to that region in the pFN fragment they used, it is likely that the activity they described may be attributable to the V segment. This suggests that our observation of the effects of the V segment on B lymphocytes is widespread and may perform some important role in vivo. While this paper was under review, a paper by Wayner et al. (1989) reported that lymphoid cells can attach to FN via interaction of a different integrin, $\alpha_6\beta_1$, with CSI (V25) peptides, which is in agreement with our own recent results (Guan and Hynes, 1990). The recombinant FNs we describe here are now being used to investigate this system in greater detail. It is clear that it will be very fruitful to explore the effects of various recombinant FNs on other specialized cellular systems both in vitro and in vivo, especially with cells lacking the classic FN receptor, $\alpha_6\beta_1$ integrin. It is likely that inclusion of more than one of these segments may change the properties of FN in some of these assays. Experiments testing these ideas are now in progress.

We would like to thank Jean Schwarzbaeuer for vectors, clones, and advice and Gene Huh, Eugene Marcantoni, and Pam Norton for helpful comments on the manuscript. Antibodies were generously provided by M. Chiquet and cells were provided by I. J. Fidler, F. Grinnell, and D. Schatz. Finally we thank the Cell Culture Center at Massachusetts Institute of Technology for growing the bulk cultures of producer cell lines.

This work was supported by a grant from the U.S. Public Health Service, National Cancer Institute (PO1CA26712). Jun-Lin Guan is a fellow of the Anna Fuller Fund and Richard Hynes is an Investigator of the Howard Hughes Medical Institute.

Received for publication 5 July 1989 and in revised form 26 September 1989.

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