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Efficient and stable expression of recombinant fibronectin polypeptides

(retroviral vectors/alternative splicing)

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ABSTRACT **We describe retroviral expression vectors containing cDNAs encoding part of fibronectin preceded by the signal and "pro" sequences of parathyroid hormone. The recombinant retroviruses were used to generate NIH 3T3 cell lines stably producing functionally active fragments of fibronectin. The recombinant fibronectins (deminectins) are processed and secreted by the cells and form disulfide-bonded dimers with themselves and with endogenous fibronectin subunits. The fibronectin-deminectin heterodimers are incorporated into the extracellular matrix. We describe cell lines producing six variant forms of deminectin corresponding to variant forms of fibronectin produced by alternative splicing. In constructing fibronectin cDNAs encoding the six variant forms, we also made use of the ability of retroviral vectors to generate cDNAs by accurate splicing of cloned genomic segments. These constructs should be valuable in analyses of the structure–function relationships of fibronectins.**

The combination of cDNA clones and eukaryotic expression vectors allows the study of protein function *in vivo*. The functional complexity and structural simplicity of the large, extracellular glycoprotein fibronectin (FN) make it particularly interesting for this type of analysis. FN has binding sites for cells, heparin, fibrin, and collagen and is involved in cell adhesion and migration, cell morphology, hemostasis, thrombosis, and oncogenic transformation (1–3). The unusual repeating structure of FN is composed of three types of homologous units: types I, II, and III. However, at least two regions of protein variability have been identified (4, 5). Analyses of cDNA clones have shown that two different patterns of alternative splicing occur within the 3′ half of the transcript of the single FN gene. At one site, a single 5′-splice site can be spliced to any one of three 3′-splice sites (4, 6), allowing inclusion of 0, 95, or 120 amino acids, at a point 30 kDa from the C terminus. These segments are not homologous with any of the repeating units of FN. The second pattern, exon skipping, allows inclusion or deletion of a single exon encoding a single, 90 amino acid type III repeat (5, 7, 8). Together, these two positions of alternative splicing can produce six possible variants in rat FN. These variations account for differences between the subunits of plasma and cellular FNs (9, 10).

A direct approach to determining the significance of these variant forms of FN in interactions with cells and extracellular matrix components is to express individual cDNAs in cultured cells and look for differences in activity of the various polypeptides produced. Several eukaryotic expression vectors have been described, but we chose retroviral-based vectors for their versatility (11) because of the following reasons: (*i*) recombinant retroviruses produced following transfection of the ψ2 packaging cell line (12) with a retroviral vector can be used to infect many cell types, (*ii*) a DNA copy of the retroviral RNA genome is stably integrated into the chromosomal DNA of the infected cell, and (*iii*) introns are removed from gene segments within retroviral vectors during production of recombinant viruses (13, 14).

The cDNAs used in this study lack the N-terminal half of FN including the signal sequence required for secretion. Therefore, a cDNA fragment encoding a heterologous signal sequence from preproparathyroid hormone (15) was fused to the 5′ end of the FN sequences. We elected to introduce these recombinant fibronectin genes into cells that already express FN so that the function of the expressed proteins could be analyzed in an appropriate context. We describe efficient synthesis and secretion of recombinant, truncated FN polypeptides (deminectins, DN). These DNs are functional in that they can form dimers with the endogenous FN molecules, and these heterodimers are incorporated into the extracellular matrix.

MATERIALS AND METHODS

Vector Construction and FN cDNAs. The expression vector, pDOP (see Fig. 2*A*), was constructed from pMSV-gpt (12) by replacement of the sequences from the *Kpn* I site at the 3′ end of the 5′ long terminal repeat to the *Xho* I site (12) with those from a murine leukemia virus-based vector (DO1; ref. 16). pDOP contains a unique *Bam*HI cloning site followed by a fragment of pBR322, the simian virus 40 (SV40) origin and early promoter, and the *neo*^{r} gene (14, 16). The *neo*^{r} gene product confers resistance to the antibiotic, G418, in mammalian cells (17). The polyoma virus early region increases the plasmid copy number after transfection into ψ2 cells.

FN cDNAs were isolated from a rat liver λgt11 library (4). *Bam*HI and *Bcl* I linkers were added to the 5′ and 3′ ends, respectively, of an *Eco*RI partial–*Sac* II fragment of λrlf 3 containing the 3′-terminal 2400 bases of coding sequence including the 360-base variable segment (V120) plus 169 bases of the 3′-untranslated region. A 110-base-pair *Bam*HI–*Bgl* II fragment from pPTHm127 (15) containing the 5′-untranslated and "prepro" coding sequences of parathyroid hormone was then ligated to the 5′ end of the linkered FN cDNA. The hybrid cDNA was inserted into the *Bam*HI site of pDOP. The sequence across the prepro-FN junction was confirmed (18). cDNAs containing the 285-base difference sequence (V95) or no additional sequences (V0) were constructed by replacing the 360-base sequence with the corresponding fragments from λrlf 4 and λrlf 6, respectively (4).

Derivation of cDNA Clones from Genomic DNA. FN cDNA containing the EIII segment was obtained by passage through

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Abbreviations: FN, fibronectin; DN, deminectin; SV40, simian virus 40; G418^r, G418-resistant; 2D, two-dimensional.

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ψ2 cells of a retroviral vector, DOL (16), carrying an 8-kilobase (kb) *Eco*RI–*Bam*HI fragment of the FN genomic clone λrFN 2 (ref. 6 and see Fig. 1*B*). After infection of 3T3 cells with recombinant virus, the G418-resistant ($G418^r$) cells were fused with COS cells to induce replication from the SV40 origin, and the provirus was rescued (14). Recovered cDNA copies of this region of the FN gene were sequenced (18), and all splice junctions were correct.

Cell Culture and DNA Transfections. 3T3 and ψ2 cells were grown in medium plus 10% (vol/vol) calf serum. COS cells (19) were maintained in 10% (vol/vol) fetal calf serum. DNA transfections were performed using calcium phosphate precipitation (20, 21). Twenty hours after glycerol shock, ψ2 medium containing transiently produced recombinant virus was removed and filtered, and 1 ml of this virus stock was used to infect 3T3 cells in the presence of polybrene at 8 μg/ml. Several days later cells were cultured in medium containing G418 at 0.5 mg/ml. The number of $G418^r$ 3T3 colonies obtained ranged from 50 to several hundred; a subset of these was isolated and expanded for further analysis. In addition, $G418^r$ ψ2 clones were tested for virus production. Viral titers for these clones ranged from 10^3 to 10^5 $G418^r$ colony-forming units/ml of supernatant.

Metabolic Labeling, Immunoprecipitation, and PAGE. Cells were labeled for 20–24 hr with medium containing [^{35}S]methionine at 25 μCi/ml (1 Ci = 37 GBq); conditioned medium, deoxycholate-soluble, and insoluble fractions of the cells were immunoprecipitated using a rabbit anti-rat cellular FN polyclonal antiserum and goat anti-rabbit IgG as described (22). Immunoprecipitates were analyzed either with or without reduction by electrophoresis through $NaDodSO_4$/polyacrylamide gels followed by fluorography. For two-dimensional (2D) analysis, samples were electrophoresed nonreduced in $NaDodSO_4$/5% polyacrylamide tube gels, reduced *in situ*, and electrophoresed through an $NaDodSO_4$/6% polyacrylamide slab gel (23).

Immunofluorescence. Both mouse (3T3) and rat FNs were detected by a polyclonal rabbit antiserum (diluted 1:100) prepared against rat FN as described (24). Rat FN or DN were detected using the mouse monoclonal anti-rat FN M9 (a generous gift of M. Chiquet, Carnegie Institute). Primary antibodies were detected by fluoresceinated or rhodaminated goat antibodies to rabbit and mouse immunoglobulins (Cappel Laboratories, Cochranville, PA). Controls showed that the monoclonal anti-rat FN did not stain 3T3 cells and that the goat antibodies reacted only with the desired primary antibody.

RESULTS

Isolation of cDNA Clones. The polypeptide structures of the six variants of rat FN are shown in Fig. 1*A*. cDNAs encoding the V0, V95, and V120 forms were isolated from a λgt11-rat liver cDNA library (4). DNA sequencing of FN genomic clones localized the exon encoding the EIII segment (8). To prepare cDNAs containing this exon, a retroviral vector carrying an 8-kb genomic fragment was transfected into the ψ2 cell line, recombinant virus was used to infect 3T3 cells, and the provirus was rescued by COS fusion (14). Introns were removed by splicing during virus production by the ψ2 cells; thus, the provirus in the infected cells was a cDNA copy of this segment of the gene. The genomic fragment contained eight introns and nine exons encoding five type III repeats (Fig. 1*B*). The rescued proviruses were analyzed by digestion with restriction endonucleases and by DNA sequencing. Two cDNAs, 1.1 and 1.4 kb, were isolated in this manner and represent the two alternatively spliced variants of the EIII region (Fig. 1*B*). Segments containing EIII were then inserted into each of the three types of cDNA.

Preparation of DN-Producing Cell Lines. The rat FN cDNAs encode the C-terminal half of FN containing binding sites for cells, heparin, and fibrin, but lack the region encoding the signal sequence. cDNA encoding the prepro sequences of preproparathyroid hormone was ligated in the correct reading frame to the 5′ end of each of the FN cDNAs, and the hybrids were inserted into pDOP (Fig. 2*A*). Fig. 2*B* shows the series of events resulting in 3T3 cells that secrete DNs. Retroviral transcripts direct the synthesis of the DNs whereas the SV40 early promoter controls transcription of the subgenomic RNA encoding the *neo*^{r} gene product. Plasmids were transfected into the two cell lines; resultant recombinant virus was used to infect 3T3 cells, and $G418^r$ clones were picked and analyzed for DN production.

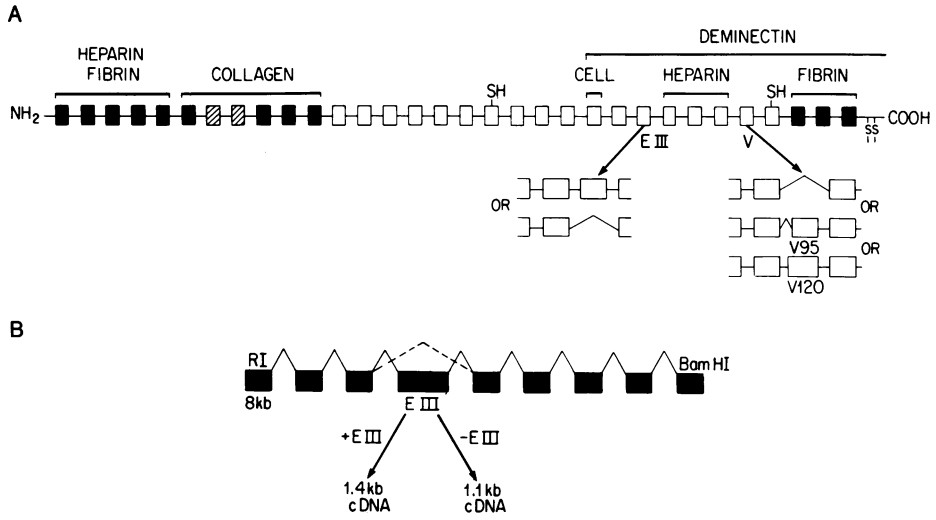

FIG. 1. (*A*) FN structure and variants. Each FN monomer consists of type-I (solid boxes), type-II (hatched boxes), and type-III (open boxes) homologous units. Interchain disulfide bonds are at the C terminus. Binding domains for fibrin, heparin, collagen, and cells are designated. The constructs used in this report contain the C-terminal 40% of FN including two regions of variation due to alternative splicing of the FN transcript. V segment, inclusion of 0, 95 (V95), or 120 (V120 = 25 + 95) amino acids between the last two type-III repeats. The V segment is not homologous with any of the repeats. EIII segment, an exon encoding one type-III repeat, is either included or omitted. (*B*) FN gene segment containing EIII. An 8-kb *Eco*RI–*Bam*HI genomic fragment was inserted into a retroviral vector and transfected into ψ2 cells. Recombinant virus was used to infect 3T3 cells, and the integrated provirus was rescued from $G418^r$ 3T3 cells by COS fusion rescue. cDNA copies +EIII (1.4 kb) and −EIII (1.1 kb) were obtained. DNA sequencing demonstrated that all splice junctions were made correctly.

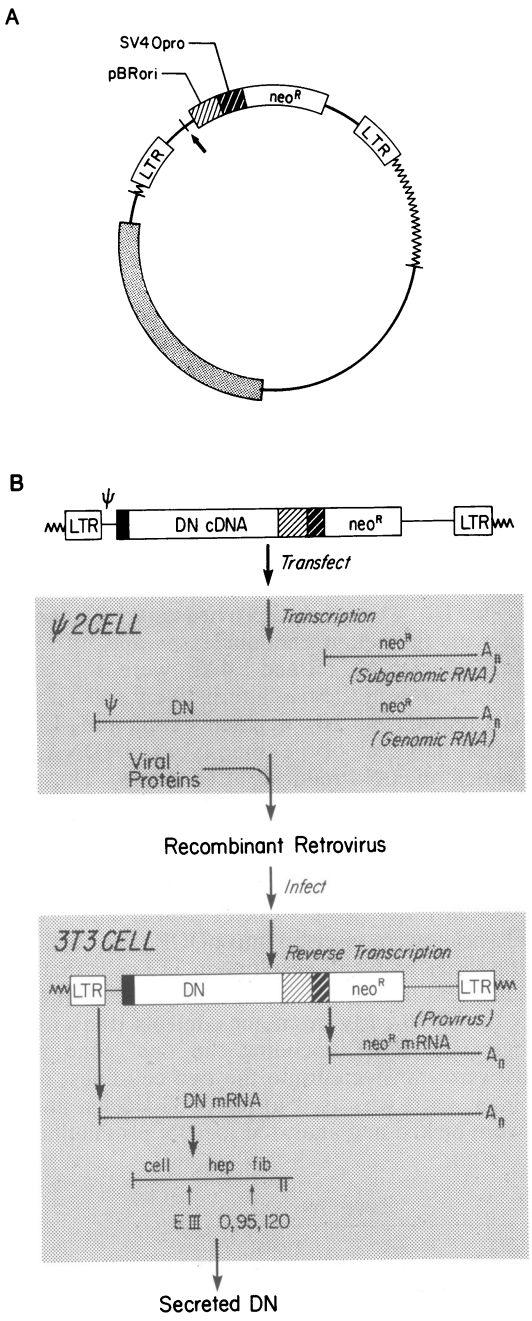

FIG. 2. (*A*) Structure of pDOP. The SV40 early promoter directs transcription of the neo^r selectable marker. The SV40 origin (within the SV40 promoter segment) and the pBR322 origin are needed for DNA replication after fusion with COS cells and in bacteria, respectively. The arrow designates the *Bam*HI cloning site into which FN cDNA segments (DN) were inserted. The provirus plus flanking mink cell DNA (∿) is within a pBR322 "poison minus" plasmid (thin line) that also contains the polyoma early region (stippled area). (*B*) Production of helper-free recombinant retrovirus and DN-producing 3T3 cells. The diagram at the top illustrates the retroviral-based portion of the recombinant pDOP used for transfection. The sequences between the 5′ and 3′ murine sarcoma virus long terminal repeats include the ψ sequence required for genomic RNA packaging, FN cDNA preceded by the signal and pro cDNA from parathyroid hormone (solid box), pBR322 origin (thin hatching), and the SV40 origin and early promoter (thick hatching). The plasmid is transfected into the ψ2 cells where it is transcribed from the promoter in the 5′ long terminal repeat, and genomic RNA is packaged into viral particles that bud out of the cells. Recombinant virus stocks are used to infect 3T3 cells where reverse transcription generates a DNA copy of the viral RNA, and this provirus integrates into the 3T3 cell genome (∿, flanking 3T3 DNA). Infected cells are G418^r and synthesize DNs that are secreted into the medium. The

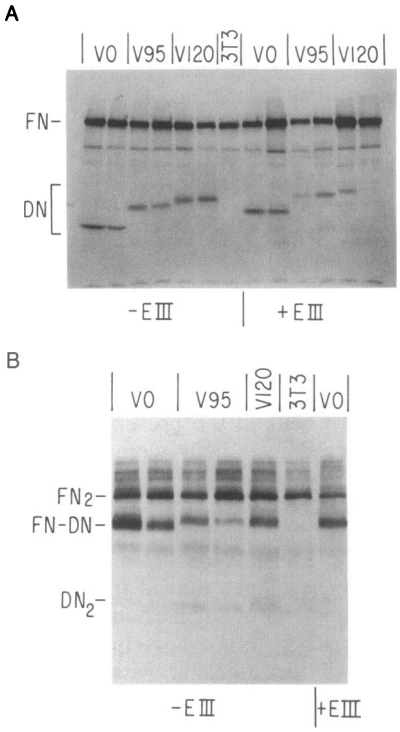

FIG. 3. One-dimensional NaDodSO$_4$/PAGE of secreted deminectins. (*A*) Culture medium from [^{35}S]methionine-labeled cells was immunoprecipitated with anti-FN antiserum. Immunoprecipitates were reduced and analyzed in a 6% gel. FN and DNs are indicated. All six variants of DN are shown: V0, V95, or V120 either with or without EIII. They range in size from 75 to 110 kDa. The 3T3 lane is from G418^r cells prepared by infection with DOP virus. (*B*) A subset of the samples in *A* but electrophoresed without reduction. The locations of FN dimers, FN-DN heterodimers, and DN homodimers are indicated. DN homodimers are apparent in V95 and V120 samples but few, if any, were detected in V0 samples. The faint band in that region of the V0 lanes is a background band present in all lanes including 3T3s.

Analysis of Secreted DNs. FN is incorporated into the matrix but it is also found in large quantities in the cell conditioned medium. The initial screening of G418^r clones involved immunoprecipitation of metabolically labeled culture medium with anti-rat FN antiserum. The heterologous signal sequence promoted efficient secretion of all variants of DN ranging in size from 75 to 110 kDa (Fig. 3*A*). The level secreted differed for each clone in this set; with the exception of V120+EIII, ratios ranged from 0.3 to 0.6 DN for each FN monomer (from densitometric data not shown).

Since the DNs contain the C-terminal cysteine residues that form the interchain disulfide bonds in FN dimers, it seemed possible that they would form dimers with each other and with the endogenous 3T3 FN monomers. Immunoprecipitates were analyzed without reduction (Fig. 3*B*). Endogenous 500-kDa FN dimers are present in all samples including the 3T3 cell control. An extra protein band migrating at about 350 kDa is present in the DN-producing clones and is approximately the size predicted for a heterodimer of FN and DN. In addition, immunoprecipitable proteins of about 200 kDa were detected in the media of the V95 and V120 lines and probably represent DN homodimers.

To identify conclusively the components of the 350- and

DNs contain domains for binding to cells, heparin, and fibrin, all permutations of the two regions of alternative splicing (EIII and V) and the C-terminal cysteines for dimer formation (diagrammed within the 3T3 cell).

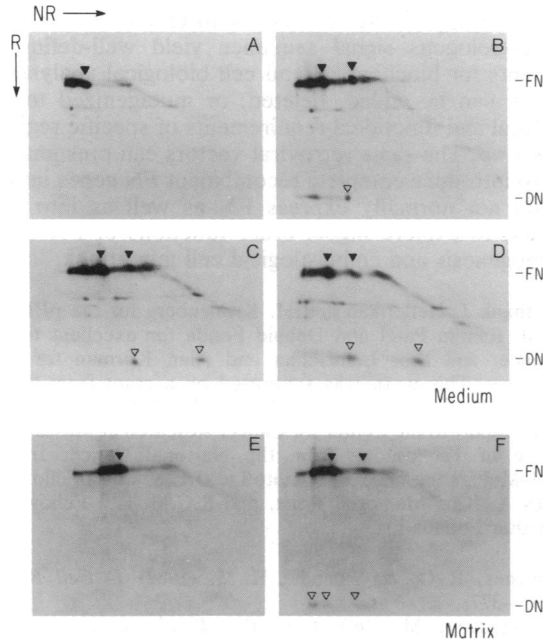

FIG. 4. Two-dimensional nonreduced (NR)-reduced (R) gel electrophoresis of deminectins. Immunoprecipitates of culture media from 3T3 (*A*), V0-EIII (*B*), V95-EIII (*C*), and V95+EIII (*D*) cell lines were separated without reduction on a $NaDodSO_4$/5% gel, and dimer components were analyzed by reduction, electrophoresis through a 6% gel, and fluorography. The locations of the FN (▼) and DN (▽) monomers are marked. DNs are clearly visible as components of heterodimers (*B–D*) and homodimers (*C* and *D* only). Higher molecular weight aggregates are present in all samples and contain both FN and DN. Immunoprecipitates of deoxycholate-insoluble material from 3T3 (*E*) and V0-EIII (*F*) cell monolayers show the presence of FN-DN heterodimers in the matrix. This was the case with all cell lines analyzed. As in the medium, high molecular weight aggregates also contain DN.

200-kDa proteins, we analyzed the samples by 2D electrophoresis. The first dimension was electrophoresed nonreduced (NR); the samples were reduced *in situ* and electrophoresed through a second-dimension gel (R). Fig. 4 *A–D* shows several examples of these 2D gels and clearly demonstrates that the 350-kDa band consists of one FN monomer and one DN monomer while the 200-kDa band represents a DN homodimer (Fig. 4 *C* and *D*). DN homodimers were not detected in the media from V0 cell lines (Fig. 4*B*). DNs are also present in the high molecular weight aggregates that do not migrate into the first-dimension gel (Figs. 3 and 4 and unpublished results). These data show that the DNs are able to form dimers and probably higher oligomers both with each other and with full-length FN monomers.

Incorporation into the Extracellular Matrix. Most matrix FN is insoluble in deoxycholate (24), so the monolayer was extracted with deoxycholate and insoluble material was dissolved in $NaDodSO_4$, immunoprecipitated, and electrophoresed on a 2D gel. In all cases, FN-DN heterodimers were present in the deoxycholate-insoluble material; one example is illustrated in Fig. 4*F* and compared with a 3T3 cell control (Fig. 4*E*). Pretreatment of the monolayer with trypsin ablated the heterodimers (data not shown) demonstrating that the deoxycholate-insoluble material is extracellular. DN homodimers were not detected in the matrices of any of the cell lines.

We also analyzed the extracellular matrices of these cell lines for DN by indirect immunofluorescence. Monolayers were fixed and stained with either a rat-specific anti-FN monoclonal antibody or an anti-rat FN polyclonal serum, which detects all FNs. DNs were detected in FN fibrils of

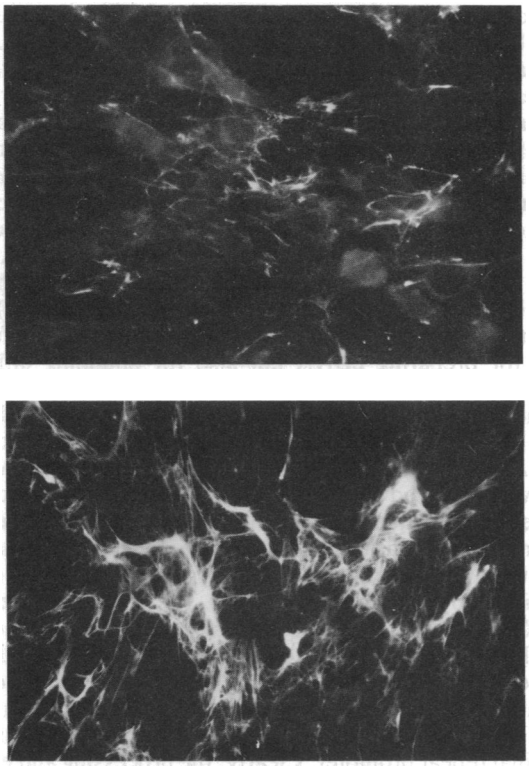

FIG. 5. Indirect immunofluorescence. V120-EIII monolayers were fixed and stained with either a rat-specific anti-FN monoclonal antibody (*Upper*) or an anti-rat FN polyclonal antiserum (*Lower*). (*Upper*) DNs are present in fibrils around and between cells. (*Lower*) All FN fibrils are stained.

DN-producing cell lines, but not in the parent 3T3 cells (Fig. 5). The level of DN incorporation into matrix appears lower than the percent secreted. From these results we conclude that these truncated FN polypeptides are incorporated into the matrix but only as heterodimers.

DISCUSSION

Two regions of variability occur in the C-terminal half of FN (3–8). These variations account for differences between the subunits of both plasma and cellular FNs (9, 10), but their structural and functional significance is not known. The variations fall within a segment of FN that contains several important binding domains for cells, heparin, and fibrin, as well as the cysteine residues required for dimer formation. Therefore, the C-terminal half of FN, when expressed *in vivo*, should retain some of these functions allowing analysis of the effects of the variant segments.

Most (95%) cellular FN subunits contain either the V95 or V120 segments while 40% of plasma FN subunits lack this region (V0) (10). On the other hand, the alternatively spliced EIII exon is included in 25–30% of the mRNAs encoding the cellular form of FN (5, 10), whereas plasma FN, synthesized by liver cells (25), lacks this segment (10). Consequently, although the FN cDNAs we isolated from the λgt11-rat liver cDNA library span the region containing EIII (4), they do not contain this exon. Therefore, we took advantage of a feature of the retroviral life cycle, splicing of the viral transcript, to generate a cDNA containing EIII from a genomic clone. All introns that are constitutively removed during normal splicing of the FN gene transcript were always removed. It is noteworthy, however, that the splicing of the two introns around the EIII exon, which varies both between cells and within a given cell type, also varies in this construct that contains only a portion of the gene, lacking both the 5′ and

3′ ends. As would be expected for 3T3 cells that make cellular FN, both "+" and "−" EIII cDNAs were obtained. This indicates that whatever determines the special behavior of this exon is contained in the transcript of this region and is not dependent, for example, on choice of promoter or poly(A) addition site. A similar conclusion has been drawn from a transient expression experiment using a portion of the human fibronectin gene (7). We have found this approach to be both efficient and accurate in that all cDNAs prepared thus far have been properly spliced. Three kilobases of cDNA have been prepared from genomic clones spanning 30 kb of the FN gene (J.E.S., R. Patel, and R.O.H., unpublished results). Our results demonstrate that this approach is generally useful not only for preparing cDNA but also for obtaining different alternatively spliced forms.

The following six variants of rat FN were analyzed: V0, V95, and V120 either containing or lacking EIII. Since the cDNAs are not full length, the FN signal sequence is absent and was replaced by cDNA encoding the parathyroid hormone signal sequence. The quantity of DN produced by different clones varies, probably due to the chromosomal site of integration (26), since the $G418^r$ clones harbor a single proviral copy (unpublished results). The levels of expression of DNs were up to 60% of the endogenous FN production, which itself represents 0.1–0.2% of total protein synthesis. Therefore, the recombinant DN genes, with heterologous initiator codon, signal, and pro sequences, function very efficiently under the control of the murine sarcoma virus long terminal repeat promoter. Clearly, the processing and export of the recombinant proteins also proceeds efficiently since large amounts of DN are secreted and since we did not observe intracellular accumulation (data not shown).

In all cases, $G418^r$ 3T3 cells secreted the DNs in the form of heterodimers with endogenous full-length FN. Therefore, if any signals for dimer formation exist, they reside within the C-terminal half of FN. In addition, densitometric analyses suggest that, with V95 and V120 DNs, the relative proportions of FN-DN heterodimers and DN homodimers are consistent with random assortment of subunits. V0 DNs are apparently incapable of accumulating significant amounts of homodimers and, in these clones, proportionately greater amounts of heterodimer are secreted. This result may suggest that part or all of the V segment is required for dimer formation or stability. However, it is clear that neither the V segment nor the EIII segment needs to be present in both chains of a dimer since $EIII^-$ and V0 DNs readily form heterodimers.

We have also shown that the DNs retain some of the functions of FN itself. Not only do they form dimers but also the heterodimers are incorporated into the matrix. DN homodimers are not incorporated suggesting that regions in the N-terminal half of FN are required for interactions within the matrix, e.g., collagen binding or FN self-association. This is consistent with other analyses suggesting a role for the N-terminal region of FN in matrix assembly (27). We have not observed any dependence on the presence of the V or EIII segments for incorporation into matrix. However, these segments could be contributed by the mouse FN chains in the heterodimers. The cell lines described here express relatively high levels of DN from a single integrated provirus. It should be possible to skew further the heterodimer/FN dimer molar ratio by introducing multiple copies of FN cDNA into these cells and potentially perturb normal FN fibrillogenesis.

The importance of this approach lies in the fact that it provides a means for studying, in detail, regions of FN, and other secreted proteins, without requiring a complete cDNA clone. The relatively high levels of expression of DNs from the retroviral promoter coupled with efficient secretion via the heterologous signal sequence yield well-defined FN fragments for biochemical and cell biological analyses. Sequences can be added, deleted, or mutagenized to study structural and functional requirements of specific regions of FN *in vivo*. The same retroviral vectors can presumably be used to introduce complete recombinant FN genes into cells that do not normally express FN as well as into mouse embryos to explore higher-order functions of FN such as morphogenesis and embryological cell migrations.

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