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On the origin of species of fibronectin

(RNA splicing/protein variants/fusion proteins)

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ABSTRACT **Multiple different subunits of fibronectin are known to occur and their origin has been unclear. Recent results showing that a single fibronectin gene can give rise to several different mRNAs by alternative splicing suggested an explanation for some of this diversity of fibronectin subunits. Because the alternative splicing events occur within the coding region, the mRNAs differ in coding potential. We have prepared recombinant phage containing a rat fibronectin cDNA segment that is present in some fibronectin mRNAs and not in others. This segment was inserted in the β-galactosidase gene of λgt11, and fusion protein produced by lysogens of the recombinant phage was purified and used as immunogen. The resulting antisera recognized some subunits of rat and hamster fibronectins but not others, indicating that inclusion or removal of this segment gives rise to mRNAs that encode different fibronectin subunits. In particular, presence or absence of a 95 amino acid segment appears to account for differences in size among the subunits of plasma fibronectin, whose origin is therefore explained by alternative patterns of RNA splicing.**

Fibronectins (FNs) comprise a group of large extracellular glycoproteins composed of structurally similar but nonidentical subunits varying in size between 210 and 250 kDa (1–3). One- and two-dimensional electrophoresis have shown that each form of FN consists of a characteristic set of distinguishable subunits (4–7). For example, one form of FN, pFN, which is found in blood plasma, consists of four types of subunit which fall into two size classes differing in apparent molecular mass by about 10 kDa. The FN synthesized by fibroblasts (cellular or cFN) contains subunits not detected in pFN (6, 7). This diversity of subunits requires an explanation. Investigations of the known posttranslational modifications (7) suggest that these modifications cannot account for all the heterogeneity, raising the possibility that the subunits differ in primary sequence. Such differences could, in principle, arise from the existence of multiple genes, from alternative processing of the primary transcript of a single gene, from posttranslational cleavages, or from some combination of these mechanisms.

We have recently isolated cDNA (8) and genomic clones (9) encoding rat FNs and have shown that several different mRNAs arise by alternative splicing of the transcript of a single FN gene. Three mRNA species were found in rat liver, the source of pFN. These mRNAs differ at a point 827 bases before the termination codon by the inclusion or omission of blocks of coding sequence, 285 or 360 (285 + 75) bases long (8). Therefore, these mRNAs encode polypeptides that differ by 95 or 120 amino acids. It seemed probable that these differences could account for some of the known FN subunits. We report here the preparation of an antiserum against the 95 amino acid segment and its use to demonstrate that this segment is present in the larger subunits of pFN but absent from the smaller ones.

MATERIALS AND METHODS

Cells. The Nil.8 fibroblast line (10) was cultured in Dulbecco's modified Eagle's medium (DME) containing 5% fetal calf serum (Flow Laboratories or GIBCO). The Rat.1 fibroblast cell line (11) was cultured in DME containing 5% calf serum. Both of these lines are clonal.

Construction of FN cDNA-Containing λgt11 Phage. All enzymes were purchased from New England Biolabs except where otherwise noted.

The 1.2-kilobase (kb) *Eco*RI restriction fragment containing the extra 360-base-pair (bp) segment was excised from the rat liver FN cDNA clone λrlf-2 (8), purified by agarose gel electrophoresis, and eluted by the glass-bead method (12). This fragment then was digested to completion with *Hph* I and *Hpa* II restriction endonucleases, producing fragments of 388, 368, 249, and 180 bp. The 249-bp fragment, which extends from nucleotide 1306 (*Hph* I site) to 1554 (*Hpa* II site) (see ref. 8), was isolated. Staggered termini were filled in by using *Escherichia coli* DNA polymerase I (large fragment) and *Eco*RI linkers (dodecamer, New England Biolabs) were added by using T4 DNA ligase (8).

λgt11 DNA was digested with *Eco*RI, and terminal phosphates were removed with calf intestine alkaline phosphatase (Boehringer Mannheim) (8). After removal of excess *Eco*RI linkers from the 249-bp fragment, this fragment was ligated to the λgt11 arms. Recombinant phage were packaged and isolated as described (8). Several recombinants were used to prepare λ lysogens in *E. coli* Y1089; lysogens were screened for isopropyl β-D-thiogalactoside-dependent production of a 125-kDa fusion protein by $NaDodSO_4$/PAGE of bacterial lysates (8, 13).

Characterization of Recombinant Phage DNA. Phage DNA was isolated from lysogens as described by Maniatis *et al.* (14). The insert and flanking β-galactosidase coding sequences were excised as a single fragment by digestion with *Pvu* II, which cuts about 350 bp 5′ and 6 bp 3′ of the *Eco*RI cloning site. After purification, this fragment was digested either with *Hin*fI, which recognizes one site 30 bp 5′ of the *Eco*RI site, or with *Acc* I, which cuts within the insert. The *Hin*fI and *Acc* I fragments were end-labeled, digested with *Dde* I or *Hin*fI, respectively, and sequenced by the method of Maxam and Gilbert (15). The sequence across the 5′ boundary confirmed that the insert was in the correct reading frame and orientation in clone λrlf-95.

Purification of λrlf-95 Fusion Protein and Preparation of Antisera. A stationary-phase culture of the λrlf-95 lysogen was diluted 10-fold with fresh L broth (final OD_{600} = 0.5) and grown with shaking at 30°C for 1 hr. The temperature-sensitive λ repressor was inactivated by incubating the culture at 45°C for 15 min; isopropyl β-D-thiogalactoside (5 mM) then was added to induce fusion protein synthesis and cultures were shaken at 37°C for 3 hr.

The β-galactosidase purification protocol outlined by Miller (16) was used with slight modification to prepare fusion protein. Cells were collected from broth by centrifugation, suspended in 0.2 M Tris Cl, pH 7.6/0.2 M NaCl/0.01 M EDTA/0.01 M 2-mercaptoethanol/5% (vol/vol) glycerol, and lysed by sonication. After cell debris was removed by

Abbreviations: FN, fibronectin; pFN, plasma FN; cFN, cellular (fibroblast) FN; kb, kilobase(s); bp, base pair(s).

centrifugation, the fusion protein was concentrated and partially purified by ammonium sulfate precipitation. The precipitate was resuspended in 10 mM Tris Cl, pH 7.6/1 mM EDTA and dialyzed against DEAE-cellulose column buffer (0.2 M NaCl/0.01 M Tris Cl, pH 7.6/0.01 M EDTA/0.01 M 2-mercaptoethanol). The sample was applied to a Whatman DE52 cellulose column and the fusion protein was eluted with a 0.2–0.45 M NaCl gradient. Peak fractions were pooled, ammonium-sulfate precipitated, and further purified by preparative $NaDodSO_4$/6% PAGE. The gel was stained lightly with Coomassie blue, the fusion protein band was cut out and pulverized in liquid N_2, and pulverized gel containing 0.5 mg of protein, with complete Freund's adjuvant, was injected subcutaneously into each rabbit. Two weeks later, a second injection was given with incomplete Freund's adjuvant. Sera positive by immunoblotting and immunofluorescence (anti-95 antisera) were obtained from 2 weeks after the booster injection. Further injections led to reactivity against polyacrylamide. Such reactivity was removed by adsorption with crushed polyacrylamide gel. Rabbit antisera to rat cFN and pFN were prepared and characterized as described previously (10), using as immunogens FNs purified by gelatin-Sepharose affinity chromatography followed by $NaDodSO_4$/PAGE.

Analysis of FNs. Hamster and rat pFNs were purified by affinity chromatography on gelatin-Sepharose as described (7, 17).

For some of the immunoblot analyses, FN subunit heterogeneity was assayed in media conditioned for 48 hr by fibroblast cultures. Cells were grown to near confluence in the appropriate medium and then were switched into medium containing serum that had been depleted of bovine FN by passing the serum over a gelatin-Sepharose column. The completeness of this depletion step was monitored by immunoblot analysis of DME containing 10% fetal calf serum depleted of FN (see Fig. 2).

Tryptic digestion was carried out at a trypsin/FN ratio of 1:500 (wt/wt) in the presence of 2 mM EDTA at room temperature. FN in 0.15 M NaCl/20 mM Caps [3-(cyclohexylamino)-1-propanesulfonic acid] buffer, pH 11, was brought to neutrality (pH 7–7.5) by the addition of 0.05 volume of 1 M NH_4HCO_3. Trypsin (Sigma) then was added, and digestion was stopped at various times by the addition of soybean trypsin inhibitor (7).

Gel Electrophoresis. One-dimensional analysis of samples was carried out in $NaDodSO_4$/5% polyacrylamide gels as described (13). Two-dimensional analysis of samples was done as described (6, 7, 18).

Immunoblot Analysis of FNs. Polypeptides were transferred from $NaDodSO_4$/polyacrylamide gels to nitrocellulose (Schleicher & Schuell, BA85) for detection by immunoblot analysis (19, 20). After electrophoresis, gels were equilibrated in 20 mM Tris Cl, pH 6.8, for 1 hr. Transfer was accomplished in a BioRad Transblot apparatus at 150 mA for 15–20 hr in 20 mM Tris Cl, pH 6.8. After transfer, the nitrocellulose sheets were washed with buffer A (25 mM Tris Cl/150 mM NaCl/0.1% Tween 20/2.5 mM EDTA, pH 7.5; four changes) for 1 hr, stained for 1–2 hr in buffer A plus 0.1% India ink (21, 22), and then destained in buffer A for 10 min. The sheets then were treated with buffer B [25 mM Tris Cl/150 mM NaCl/2.5 mM EDTA/2% hemoglobin (Sigma), pH 7.5] at room temperature for at least 2 hr.

Incubation of the blots with anti-rat FN antisera or anti-95 antisera was carried out at a dilution of either 1:200 or 1:500 in buffer B at room temperature. ^{125}I-labeled protein A (New England Nuclear-DuPont) at 10^6 cpm/150 cm^2 of nitrocellulose paper was used to detect specific binding of primary antisera to FN subunits. Incubation with pre-immune sera did not result in protein A-labeling of FN subunits. The India ink staining method detected all the FN subunits previously identified by Coomassie blue staining or [^{35}S]methionine labeling (7) and allowed precise alignment with autoradiographs of the blots.

In some experiments, we observed India ink-positive material with an apparent molecular mass of 55–65 kDa (see Fig. 4) that also reacted with pre-immune sera. The detection of this material is dependent upon the presence of dithiothreitol in the electrophoresis sample buffer (see Fig. 4, lane X) and it does not represent protein.

RESULTS

We have shown previously that three FN mRNAs exist in rat liver (8) but are all derived from the transcript of a single gene (9). The purpose of the present study was to determine to what extent variations in splicing contribute to the observed heterogeneity of FN subunits. One approach to identifying which FN subunits are encoded by which of the three mRNAs is to use antibody probes. We chose to use the λgt11 system to produce β-galactosidase-FN fusion proteins as immunogens, because such fusion proteins can be produced in high abundance in bacteria (8) and can be purified easily via standard β-galactosidase purification techniques (16).

The scheme for preparation of fusion protein is shown in Fig. 1. cDNA clone λrlf-2 (which contains the sequence corresponding to the entire 360-base segment involved in alternative splicing) was digested with *Hph* I and *Hpa* II restriction endonucleases. A 249-bp fragment which contains 83 of the 95 codons within this segment was purified and inserted into the *Eco*RI site of λgt11 via synthetic *Eco*RI linkers. Recombinant phage were used to prepare λ lysogens and several lysogens were screened for production of a 125-kDa fusion protein. The recombinant DNA from positive clones was sequenced to confirm that the insert was in the correct orientation and reading frame within the β-galactosidase gene. Fig. 1 shows the nucleotide and amino acid sequences across the boundaries of β-galactosidase with the 5′ and 3′ ends of the FN cDNA. At both junctions, the *Eco*RI linkers contribute an additional amino acid not found in either β-galactosidase or FN, and the two amino acids [glutamic acid (E) and phenylalanine (F)] encoded by the *Eco*RI site itself are duplicated. Furthermore, the FN cDNA insert remains in-frame with β-galactosidase across the 3′ boundary, thus producing a fusion protein that ends with the actual β-galac-

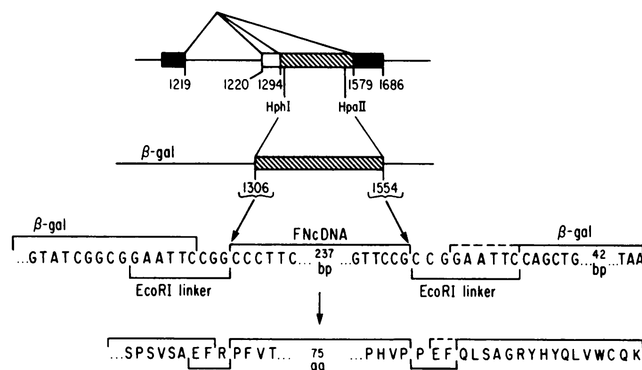

FIG. 1. Construction of β-galactosidase-FN fusion protein. The complex FN exon is spliced in three different ways as shown (8, 9). Blocks of coding sequence found in all mRNAs are shaded, whereas the 285- and 75-base segments, which are present in only some mRNAs, are hatched and unshaded, respectively. Numbers refer to nucleotide positions in the rat FN cDNA sequence (8). A 249-bp segment between *Hph* I and *Hpa* II sites was purified from the cDNA clone λrlf-2 (8), *Eco*RI linkers were attached, and the fragment then was inserted into the *Eco*RI site of the β-galactosidase gene of λgt11 (23). Nucleotide sequences across the boundaries were determined (15) and are shown, as is the amino acid (aa) sequence of part of the fusion protein encoded by the recombinant gene. Single-letter amino acid notation is used (24).

tosidase carboxyl terminus.

The fusion protein was purified from bacteria by the standard β-galactosidase purification protocol (16), with a yield of 5–10 mg per liter of bacterial culture, and used to immunize two rabbits. Antisera (anti-95) were tested for reactivity by immunoblotting. Sera from both animals were reactive with both β-galactosidase and FN (see below).

Fig. 2 shows an immunoblot of a one-dimensional $NaDodSO_4$/polyacrylamide gel of rat and hamster pFNs and cFNs probed either with anti-95 antiserum or with a polyclonal anti-rat FN antiserum. Rat pFN and cFN and hamster pFN each consist of at least two subunits that differ in size by about 10 kDa; hamster cFN is composed of at least three monomers but does not contain a subunit equivalent to the smallest subunit present in the other three FNs (7). The anti-FN serum recognizes all of the subunits (Fig. 2, lanes 1 and 2). In contrast, although the anti-95 serum reacts with all hamster cFN subunits, it does not react with the smallest subunit present in hamster or rat pFN or in rat cFN (Fig. 2, lanes 4 and 5).

Immunoblot analysis of FN after two-dimensional electrophoresis was used to confirm the identity of the subunits that contain the 95 amino acid segment. All FN subunits were detected by staining the nitrocellulose replicas of the two-dimensional gels with India ink (21, 22). This method has the advantage of allowing direct comparison and alignment between the stained pattern (indicative of the total protein sample) and the immunoblot autoradiograph (indicative of polypeptides in the sample that are recognized by the antiserum).

Fig. 3 shows the India ink-stained patterns (*A*, *C*, *E*, and *G*) of rat and hamster FNs and the respective autoradiographs (*B*, *D*, *F*, and *H*) made after labeling with the anti-95 serum and ^{125}I-labeled protein A. Whereas all of the isoelectric variants of hamster cFN are recognized (compare *G* with *H*), only the more slowly migrating isoelectric variants of rat

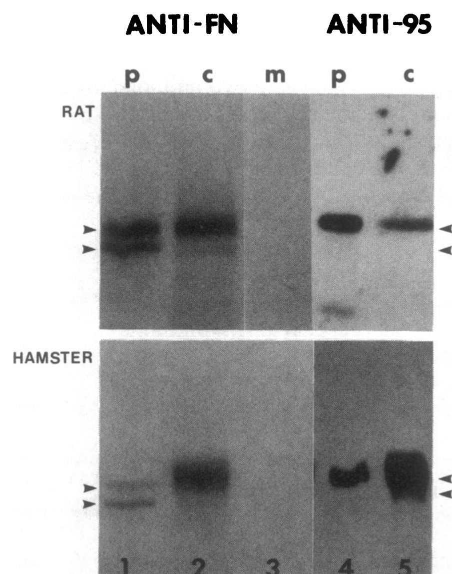

FIG. 2. Immunoblots of FN subunits. Samples were resolved by $NaDodSO_4$/5% PAGE, transferred to nitrocellulose filters, and stained with rabbit antisera against rat FN (anti-FN) or against the β-galactosidase-FN fusion protein described in Fig. 1 (anti-95). Bands binding ^{125}I-labeled protein A then were visualized by autoradiography. Lanes: p, rat or hamster plasma (containing pFN); c, culture medium conditioned by Rat.1 or Nil-8 hamster cells (containing cFN); m, unconditioned culture medium containing FN-free fetal calf serum (10% vol/vol). Whereas anti-FN stains two bands in pFN and at least two in cFN, anti-95 stains only a single band in pFN and fails to stain the smallest subunits in rat pFN, rat cFN, and hamster pFN. Arrowheads mark the positions of the pFN subunits that were stained with India ink.

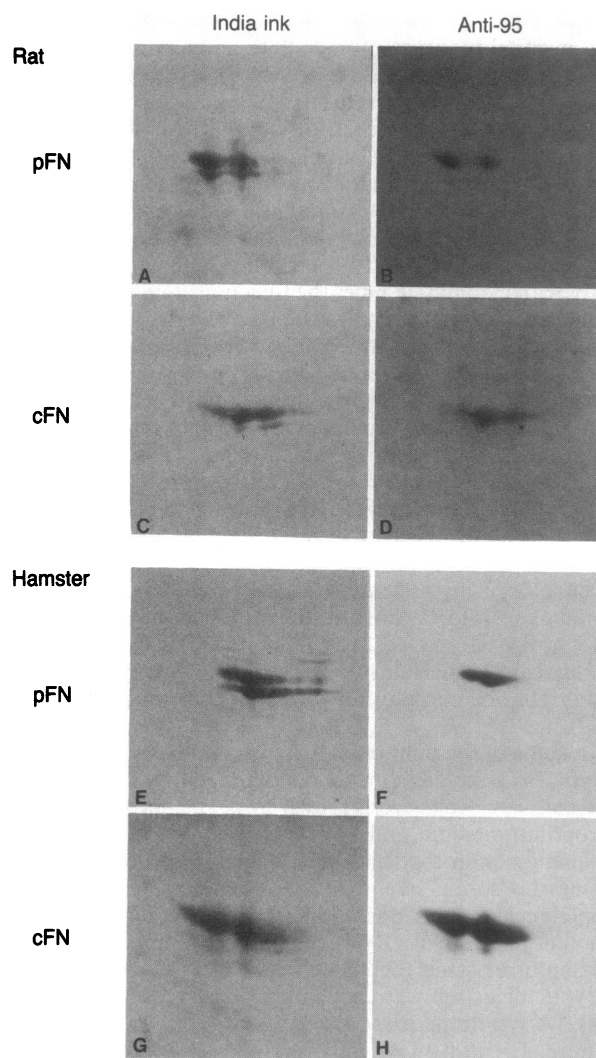

FIG. 3. Two-dimensional analysis of FN subunits. FNs purified by gelatin-Sepharose affinity chromatography were separated on two-dimensional gels and transferred to nitrocellulose filters. Transferred proteins were stained with India ink (*A*, *C*, *E*, and *G*) and then with antiserum against the fusion protein (anti-95) followed by ^{125}I-labeled protein A (*B*, *D*, *F*, and *H*). For each type of FN, all the higher molecular weight subunits stain with anti-95 but the lower molecular weight subunits of rat pFN and cFN and hamster pFN do not stain. The minor spots seen in *H* are the result of a small amount of degradation during purification of hamster cFN in this experiment.

pFN and cFN and hamster pFN are recognized by this antiserum. Therefore, the 95 amino acid segment is absent from the smallest subunits of pFN in both rats and hamsters but present in the larger subunits. This segment is present in all subunits of hamster cFN and in all but the minor lower molecular weight subunits of rat cFN.

Limited proteolytic digestion of FNs with trypsin gives rise to a characteristic array of fragments (Fig. 4). Fig. 4*A* shows a time course of tryptic digestion of rat cFN; *B* and *C* show immunoblots of the products of similar digestions probed with the anti-95 serum. Only the 40-kDa fragment (and a short-lived 200-kDa early tryptic fragment) is recognized by this serum. This 40-kDa fragment has previously been located in the subunits of FN (see refs. 7, 25, 26 and *Discussion*).

DISCUSSION

It is clear from the results presented here that the difference in size between the subunits of plasma fibronectin, which

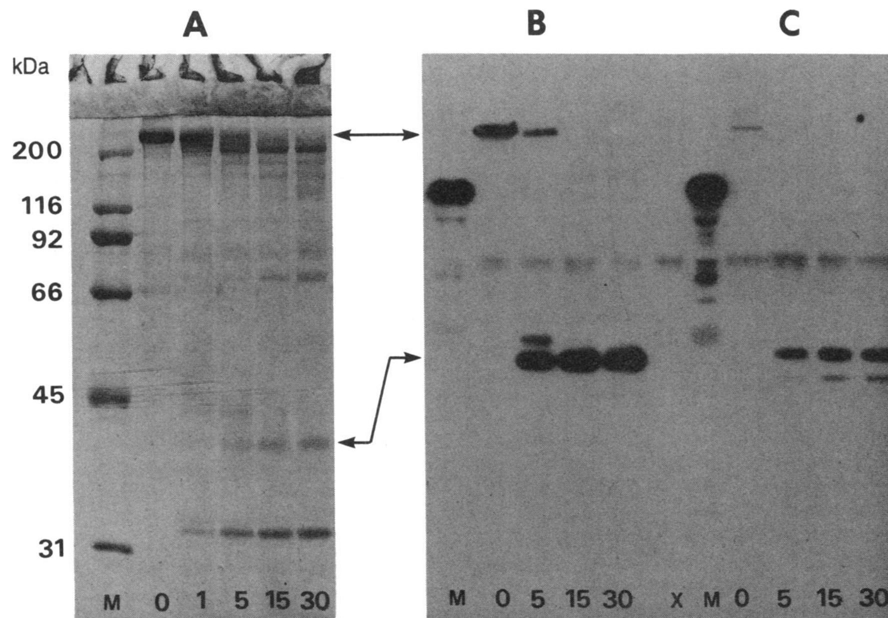

FIG. 4. Location of the 95 amino acid segment. (*A*) Coomassie blue-stained gel showing time course of tryptic digestion of rat cFN; minutes of digestion with FN/trypsin ratio of 500:1 (wt/wt) are shown at the bottom. Major fragments are at approximately 200 and 30 kDa and there is a minor fragment at 40 kDa. Markers (lane M) include β-galactosidase (116 kDa). (*B* and *C*) Immunoblots, using anti-95 antiserum, of tryptic digests of rat cFN (*B*) and rat pFN (*C*). Note reaction of antiserum with β-galactosidase (and its fragments) in the marker mixture (lanes M) and with the minor 40-kDa fibronectin fragment. The stained band at about 60 kDa is also seen in the lane that contained only electrophoresis sample buffer (lane X).

has been known for some years but was previously unexplained, is caused by alternative RNA splicing within the coding sequence. Antisera raised against a stretch of polypeptide that can be included or omitted depending on the pattern of splicing (8) recognize the larger subunits of rat and hamster FNs but not the smaller subunits (Fig. 2 and 3). Although these results show that molecular weight differences between pFN subunits are due to this 95 amino acid segment, they do not explain all the pFN subunits, of which there are four (refs. 6 and 7 and Fig. 3). It seems very likely that the third alternative splice, which includes a 75-nucleotide segment as well as the 285-nucleotide segment (8), is responsible for one of the additional variants. The 25 amino acid segment included by this splicing variation is acidic and could account for a shift in isoelectric point. We have immunized rabbits with a fusion protein containing this 25 amino acid segment but have not yet obtained antisera of sufficient titer to test this hypothesis.

The antiserum to the 95 amino acid segment also reacts with human pFN, although somewhat weakly (unpublished data). A recent paper (27) presenting the sequence of a human FN cDNA clone covering this region showed that this human FN sequence contained the 25 amino acid segment but only the first 64 amino acids of the 95 amino acid segment. Inspection of the sequences of the corresponding rat cDNA (8) and genomic (9) clones reveals that the rat FN gene could not generate a shorter insertion of 89 amino acids (267 bases) analogous to that found in this human clone. However, a single base change (adenine to guanine) in the rat FN genomic sequence would generate a 5′ splice site exactly at the end of the relevant 267-base segment. Therefore, it is possible that the human FN gene has such a 5′ splice site that can lead to removal of the last 93 bases of the 360-base difference segment, producing an mRNA encoding a segment that lacks the last 31 amino acids of the 95 amino acid difference segment of rat FN. In the absence of other cDNA or genomic clones covering this region of human FN, it is unclear how many variants differing at this position are present in humans.

The results presented here also show that the great majority of cFN subunits contain the 95 or the 120 amino acid segment. The exceptions are two minor lower molecular weight subunits of rat cFN, which we have shown previously to differ from rat pFN subunits only in their glycosylation (7). Therefore, one significant difference between cFN and pFN is that, while all (or virtually all) subunits of cFN contain the 95 or the 120 amino acid segment, a sizeable proportion of the pFN subunits lack either of these segments. This difference between cFN and pFN arises from differences in the splicing of FN transcripts in the cells that synthesize these two forms of FN, fibroblasts and hepatocytes, respectively (6–8). There are, however, other differences between cFN and pFN; cFN contains subunits that are not seen in pFN (6, 7). These cFN-specific subunits are somewhat larger and more acidic than the larger subunits of pFN. As shown here, they contain the 95 or 120 amino acid segments, but this cannot account for their difference from pFN subunits; nor can glycosylation (7).

Analysis of FN cDNA clones from a human tumor cell line revealed another region of difference between FN mRNAs (28). The second region of difference consists of a stretch of 270 bp encoding 90 amino acids which is either present or absent in FN mRNAs. We have also identified this second "difference segment" in the rat FN gene (unpublished results). It is located 815 bp 5′ of the 360-bp segment. This difference segment has not been detected in the sequence of pFN subunits (29), in rat liver FN cDNA clones (8), or by nuclease S1 analysis of liver RNA (28), and it is probable that inclusion of this segment is a characteristic of cFN. It seems likely that much of the heterogeneity of FN subunits arises from facultative splicing of the primary transcript, as we have shown here for the difference between pFN subunits. As discussed elsewhere (8), alternative splicing also can account for variant forms of several other proteins.

The identification of a site of primary sequence difference between pFN subunits and between cFN and the smaller subunits of pFN explains a variety of earlier results in addition to the long-established differences in apparent molecular weight. It was reported in 1981 (26) that a monoclonal antibody, sp4, recognized hamster cFN preferentially over

hamster pFN. This antibody binds to a 40-kDa sulfhydryl-containing tryptic fragment (26) that maps to the carboxyl-terminal third of FN (25); the same fragment is also recognized by antibody to the 95 amino acid segment (Fig. 4). Therefore, the determinant recognized by sp4 lies in or near the 95 amino acid segment that is present in all subunits of hamster cFN but in only 50% of the subunits of hamster pFN (Figs. 2 and 3). This location is consistent with the difference in reactivity of this antibody with the two forms of FN. Several other papers describing fragments of FN have suggested that differences between the subunits of pFN or between pFN and cFN occur in the same region (30–35). These results are all concordant with the present demonstration of a primary sequence difference at this location. Another difference between FN subunits is also located in this region. We reported recently that the smallest subunits of rat and hamster FNs are not sulfated whereas the larger ones are (7). The sulfate is attached to tyrosine and is located in the same 40-kDa sulfhydryl-containing tryptic fragment. There are four tyrosine residues within the 95 amino acid segment and a cysteine residue just carboxyl-terminal to it (8). Therefore, the sulfation of FN subunits reflects the presence of the 95 amino acid segment and acts as a biochemical marker for it.

Finally, we should consider briefly the possible functional implications of the inclusion or omission of the FN difference segments as a result of alternative splicing. The heparin-binding region of FN is made up of several homologous repeats of about 90 amino acids, which are known as type III homologies. The 95 or 120 amino acid segments are inserted between the last two type III homologies but are not themselves related to these homologies. The inserted segments could be involved in binding heparin or hyaluronic acid, in cell adhesion, or in self-association and fibrillogenesis (8). Several of these properties have been reported to differ between pFN and cFN. The isolation of cloned cDNAs encoding and of antibodies against these segments should aid greatly the further analysis of their significance.

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