A Tumor-associated Fibronectin Isoform Generated by Alternative Splicing of Messenger RNA Precursors

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Abstract. Fibronectin (FN) represents the mixture of a number of structurally different molecules (isoforms) whose make-up varies depending on the FN sources. FN from cultured transformed human cells has a very different isoform composition with respect to its normal counterpart. In fact, SV-40-transformed WI-38VAI3 human fibroblasts produce high levels of a FN isoform (B-FN) which is very poorly expressed in their normal, WI-38, counterpart. We have recently demonstrated that the B-FN isoform derives from a differential splicing pattern of the FN primary transcript which leads, in transformed cells, to a high level expression of the exon ED-B (Zardi, L., B. Carnemolla, A. Siri, T. E. Petersen, G. Paolella, G. Sebastio, and F. E. Baralle. 1987. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2337–2342). Here we report on the production and characterization of a monoclonal antibody (BC-1) which recognizes an epitope within the protein sequence coded for by the ED-B exon. This monoclonal antibody makes it possible to carry out immunohistochemical analysis of the distribution of the ED-B-containing FN isoform (B-FN) in human tissues. The results show that while in normal, adult, human tissues total FN has a widespread distribution, the B-FN isoform is restricted only to synovial cells, to some vessels and areas of the interstitium of the ovary, and to the myometrium. On the contrary, the B-FN isoform has a much greater expression in fetal and tumor tissues. These results demonstrate that, in vivo, different FN isoforms have a differential distribution and indicate that the B-FN isoform may play a role in ontogenesis and oncogenetic processes.

FIBRONECTIN (FN)[1] is a polymorphic high molecular mass adhesive glycoprotein present in soluble form in plasma and other body fluids and in insoluble form in the extracellular matrixes. Both these forms are dimers composed of subunits with a molecular mass of 250–280 kD made up of a series of repeating units of three types and joined by two disulfide bonds at the carboxyl terminus of the molecule. FN molecules are involved in various biological phenomena including the establishment and maintenance of normal cell morphology, differentiation, wound healing, cell migration, and adhesion (1, 2, 15, 41).

It has been previously demonstrated that FN polymorphism is at least partially caused by alternative splicing schemes in three regions of the primary transcript of a single gene which may generate 20 different FN subunit isoforms (18, 19, 29). One of these regions (IIICS, see Fig. 1 *A*) is between the last two type III homology repeats; a single exon is subdivided to yield five alternative patterns of splicing. Hynes and co-workers (34) showed that inclusion of the IIICS sequence contributed to the differences in size between the larger and smaller subunit of plasma FN. Humphries et al. (16) suggested that for some cell types, regulation of the adhesion-promoting activity of FN may occur by alternative RNA splicing in the IIICS area.

At the second region of variation (ED-A), a single exon encoding a complete type III repeat is either included or omitted from the mature mRNA. This variation is tissue specific and the ED-A sequence is absent in the mRNA of liver (10, 20, 21) which is the source of plasma FN (38). Using a rabbit antiserum to the rat ED-A segment, Paul et al. (31) demonstrated that this sequence is not present in plasma FN but is expressed in FN released by cultured fibroblasts and in FN from blood platelets. More recently, using mAbs Castellani et al. (8) and Borsi et al. (5) demonstrated that in FN from the tissue-culture medium of tumor-derived or SV-40-transformed human cells, the percentage of FN molecules containing the IIICS and ED-A sequences are higher than in FN from normal human fibroblasts.

At the third region of variation (ED-B), a single exon encoding a complete type III repeat is either included or omitted from the mature mRNA (14, 33, 44). The ED-B sequence presents two interesting peculiarities: (*a*) it is the more conserved FN region, 100 and 96% homology with rat and chicken FN, respectively (28, 44); and (*b*) this exon is highly expressed in transformed human cells, while it is barely de-

1. *Abbreviation used in this paper*: FN, fibronectin.

tectable in cultured normal human fibroblasts (44). These observations suggest that the ED-B sequence may introduce some specific biological function(s) into the FN molecule. A first step towards the understanding of the biological function(s) of the B-FN isoform is the study of its distribution in normal, pathological, and fetal tissues. In this paper we report the production and characterization of an mAb (BC-1) specific for the protein sequence coded for by the ED-B exon. Using this mAb we demonstrated that while this sequence is undetectable in plasma FN and in almost all normal human adult tissues, it is highly expressed in fetal and tumor tissues.

Materials and Methods

Cell Lines and Monoclonal Antibodies

All cell lines were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, Scotland, UK). When FN had to be quantified in conditioned media, fetal calf serum was depleted of bovine FN by passage through a large capacity gelatin-Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden). mAbs specific for human FN were prepared as previously described (43) by fusion of P3U1 myeloma cells with splenocytes from mice immunized with FN from WI38VA13 or WI38 cells, or plasma. The characterization of mAbs IST-4 and IST-9 has been previously reported (5, 7, 36). The mAb 3E3 (32) specific to the cell-binding region of FN was a gift from M.D. Pierschbacher and E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA). The radioimmunoassay was carried out as previously reported (45) and immunodot-blot analysis was performed using Bio-Rad Laboratories (Richmond, CA) equipment following the manufacturer's instructions.

Purification and Proteolytic Digestion of FN and Its Fragments

FN was purified from human plasma and from the conditioned media of cell lines as previously reported (46). Thermolysin (Protease type X; Sigma Chemical Co., St. Louis, MO) digestion of FN and cleavage of FN by *S*-cyanilation with 2-nitro-5-thiocyanobenzoic acid were carried out as described by Sekiguchi and Hakomori (35). The FN 120- and 110-kD fragments (domain 4, see Fig. 1 *A*) were purified from a FN–thermolysin digest (6 μg/mg of FN for 2 h at 22°C) by a hydroxyapatite (DNA grade; Bio-Rad Laboratories) chromatography column as previously described (6, 42). Complete separation of the 120-kD domain 4 from the 110-kD domain 4 was achieved using a DEAE-cellulose (Whatman Inc., Maidstone, England, UK) chromatography column. The two fragments, 35 and 85 kD, obtained by thermolysin digestion of the 120-kD domain 4 were separated by a hydroxyapatite chromatography column. SDS-PAGE and immunoblotting were carried out as described by Laemmli (22) and Towbin et al. (39).

Acrylamide, SDS, and other electrophoresis reagents were from Bio-Rad Laboratories. Nitrocellulose filters were from Schleicher and Schuell, Inc. (Dassel, West Germany). Rabbit antiserum to human FN was prepared as previously described (46).

Construction of cDNA Library in λgt 11 Phage

Total RNA was extracted from WI-38VA13 cells following the procedure of Chirgwin et al. (9) as modified by Freeman et al. (12). Poly(A) RNA was purified using an oligo(dT)-cellulose column according to the method described by Maniatis et al. (24). For cDNA preparation, a cDNA synthesis system purchased from Pharmacia Fine Chemicals was used following the manufacturer's instructions. cDNA, without further purification, was treated with Eco RI metilase (BioLabs, Beverly, MA), Eco RI linkers (BioLabs) were added, and the cDNA digested with Eco RI. cDNA was then ligated to λgt 11 arms using Stratagene (San Diego, CA) kits, for both ligation and packaging, following methods described by Huynh et al. (17). All enzymes used, unless otherwise specified, were from Boehringer Mannheim GmbH (Mannheim, West Germany).

Isolation and Analysis of FN cDNA Clones

A WI-38VA13 cDNA library, in the expression vector λgt 11, prepared as described above, was screened by an immunoenzymatic procedure (17) using the mAb BC-1 and an immunoenzymatic kit purchased from Promega Biotec (Madison, WI). Bacteriophage DNA was prepared from positive clones, and the Eco RI inserts were excised, subcloned into a pUC8 plasmid vector (11, 40), and sequenced by Maxam and Gilbert's method (27). All the positive clones obtained contain a FN insert with at least part of the ED-B sequence. One of these clones, λF2, contains the complete ED-B sequence and 315 bases upstream and 255 bases downstream. We constructed a clone λF6c by removing the region containing the ED-B sequence from the clone λF2 by Bsp MI and Ban II restriction enzymes (BioLabs) and substituting it with an identical fragment, except that it lacked the ED-B sequence. This fragment was obtained by the same restriction enzymes from the clone pFH154 (20) in which the ED-B sequence is not present. The clone pFH154 was a gift from Dr. F. E. Baralle (Istituto Sieroterapico Milanese, Milan, Italy). All the cloning and subcloning procedures were carried out according to Maniatis et al. (24).

For the immunoblotting analysis the β-galactosidase–FN fusion proteins were obtained from the clones using the following procedure: ~20,000 plaque-forming units were plated with *Escherichia coli* (Y 1090 strain) in 0.7% LB agar containing 0.5 mM isopropyl β-D-thiogalactopyranoside, 50 μg/ml ampicillin in 95-mm Petri dishes and incubated at 37°C overnight. Then 2 ml of Tris-HCl, pH 6.8, buffer, containing 2% SDS, 4% β-mercaptoethanol, 10% glycerol, was added to each dish and incubated at room temperature for 2 h with gentle agitation. The buffer was then removed, briefly centrifuged, and 20-μl samples of the supernatant were analyzed by SDS-PAGE.

Tissue Samples and Immunohistochemical Studies

Normal and neoplastic tissues were obtained from surgical samples of patients free from chemo- and radiotherapy. Fetal tissues were taken from spontaneous abortions. Each sample was divided into two portions: one was processed for conventional histopathological diagnosis and the other was immediately snap-frozen in liquid nitrogen. 4-μm-thick cryostat sections were stained with 0.1% toluidine blue in PBS (20 mM Na-phosphate buffer, pH 7.3, 0.15 M NaCl) and additional frozen sections were used for indirect immunofluorescence and immunoperoxidase staining after fixation in cold absolute acetone for 10 min. To avoid the heterogeneous distribution within the tissues of FN isoforms being responsible for false negative findings, at least three nonconsecutive sections of the tissue were analyzed.

All indirect immunofluorescence and immunoperoxidase stains were made using mAbs as the hybridoma culture supernatant, rabbit anti-mouse Ig FITC-labeled antiserum (Cappel Laboratories, Cochranville, PA), and a commercially available avidine-biotine staining kit (Vector Laboratories, Inc., Burlingame, CA). The immunoenzymatic stain used 3-amino-9-ethylcarbazole as a chromogenic substrate and Mayer's hematoxylin as a counterstain followed by mounting in buffered glycerol.

Results

Generation of the mAb BC-1 Which Recognizes an Epitope Within the FN Sequence ED-B

mAbs obtained by fusion of splenocytes from mice immunized using FN from the culture medium of SV-40-transformed human fibroblasts WI-38VA13 were screened with a radioimmunoassay system using plasma FN, FN from WI-38VA13 cells, and FN from their normal counterpart WI-38 cells as antigens. We obtained a clone (BC-1) which released mAbs specific for FN from WI-38VA13 cells but which did not react with plasma FN and only reacted very weakly with FN from the normal human fibroblasts WI-38. We tested this mAb using the immunoblotting technique, with thermolysin digests of FNs from plasma, WI-38, and WI-38VA13 cells. In accordance with the data obtained using the radioimmunoassay system, the mAb BC-1 showed strong reaction with WI-38VA13 FN fragments, negative reaction with plasma FN fragments, and a barely detectable reaction with WI-38 FN fragments (Fig. 1 *B*). The immunoblot pattern obtained with the mAb BC-1 was compared with those of other mAbs specific to different human FN epitopes (Fig. 1 *B*). The staining pattern obtained with the mAb IST-9

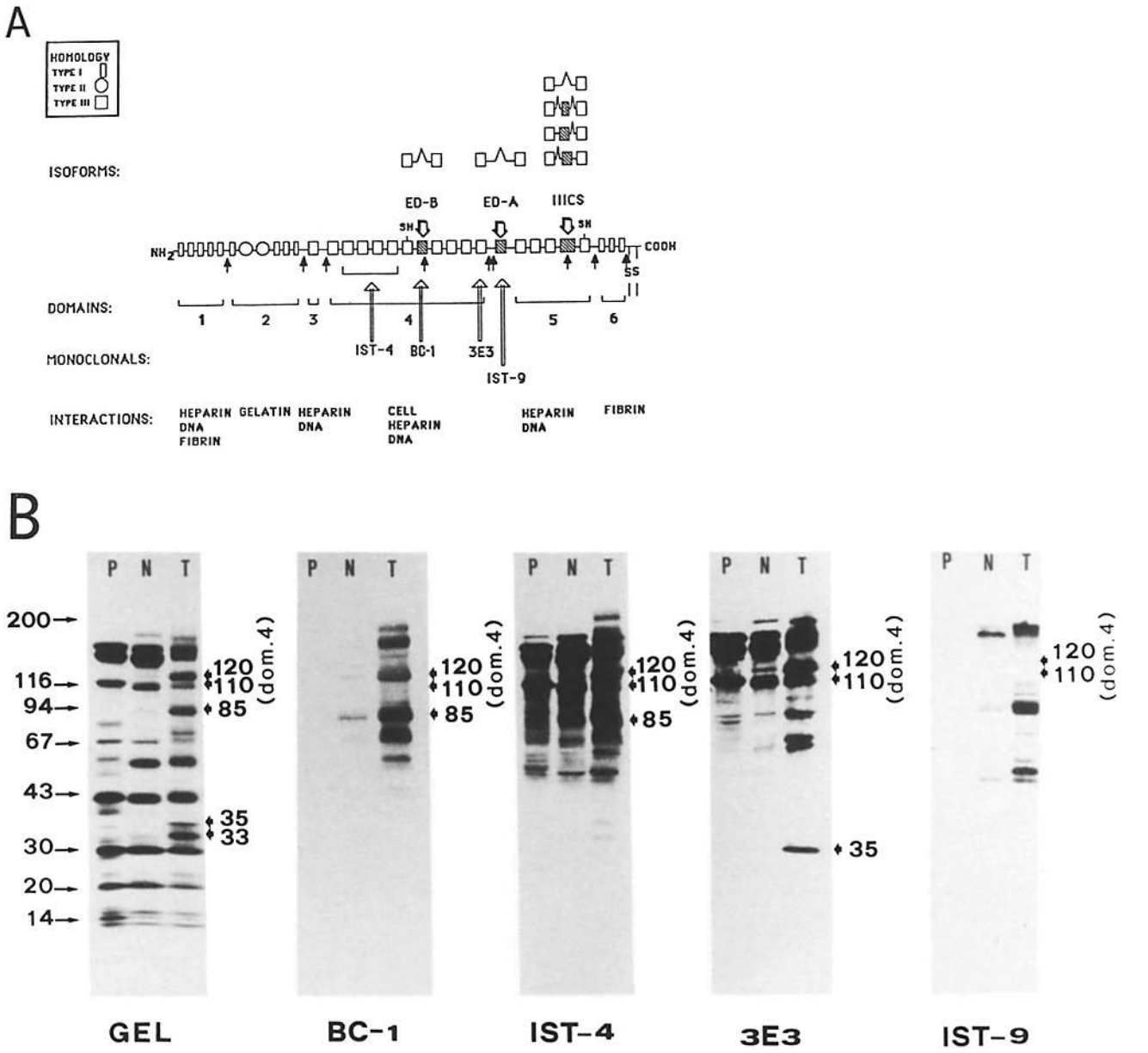

Figure 1. (A) Model of the domain structure of a subunit of human FN. Large open arrows indicate the regions of variability due to alternative splicing of the FN mRNA precursors. Black arrows indicate the thermolysin cleavage sites. Long open arrows indicate the sites where the epitopes recognized by various mAbs are located. The figure also indicates the internal homologies; macromolecules interacting with the various FN domains and the possible isoforms generated by alternative splicing. (*B*) On the left a 4–18% SDS-PAGE gradient of 20 μg each of plasma FN (*P*), FN from WI-38 normal human cells (*N*), and from SV-40-transformed human fibroblasts WI-38VA13 (*T*) digested by thermolysin (5 μg/mg of FN) for 2 h at 22°C is shown. The values on the left are the molecular masses (in kD) of the standards. The values on the right are the molecular masses (in kD) of the 110-kD domain 4 and of the four major fragments only present in thermolysin digests of FN from transformed cells. Immunoblots of a similar gel using the mAbs BC-1, IST-4, 3E3, and IST-9 are also shown. The two forms of the domain 4 120-kD fragment (containing the ED-B sequence) and 110-kD fragment (without the ED-B sequence) are indicated.

(which recognizes the ED-A sequence) was completely different from that obtained with the mAb BC-1 (Fig. 1 *B*). This rules out the hypothesis that the mAb BC-1 recognizes the ED-A sequence which is preferentially expressed in FN from transformed cells and absent in plasma FN (5). The mAb BC-1 reacts strongly with a 120-kD fragment, which is the cell-binding domain 4 containing the ED-B sequence (see Fig. 1 *B*) that is produced almost exclusively by transformed cells (44), but it does not react with the 110-kD fragment, which is the cell-binding domain 4 without the ED-B sequence (see Fig. 1 *B*). These data strongly suggested that the epitope recognized by the mAb BC-1 is within the ED-B sequence. The 110- and 120-kD cell-binding domain 4 were purified and digested with thermolysin (Fig. 2). While the 110-kD domain 4 is resistant to thermolysin, the digestion of the 120-kD domain 4 generates two fragments: a 85-kD fragment which contains almost the complete ED-B sequence in its carboxyl-terminal part and reacts with the mAb

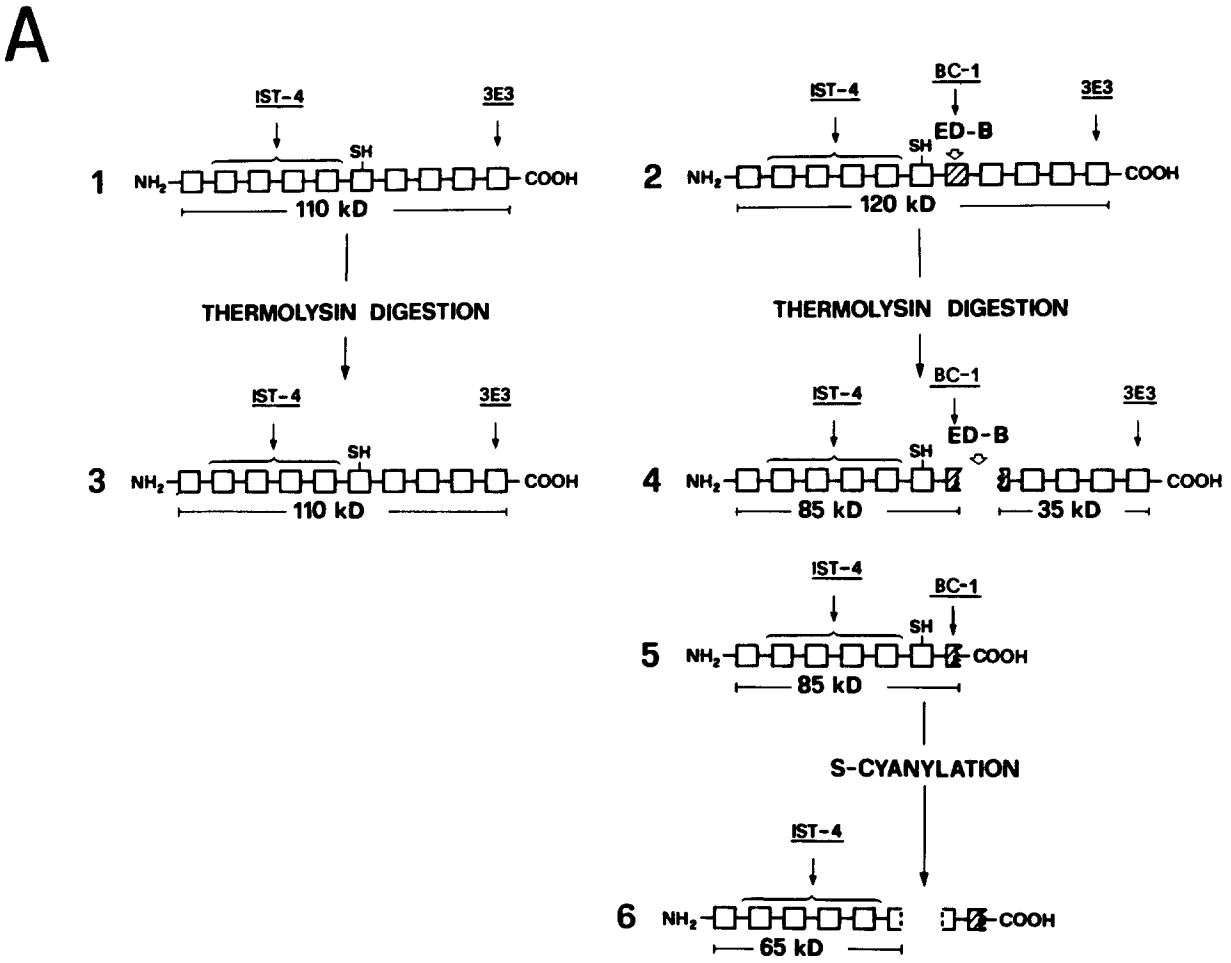

Figure 2. (*A*) Schematic representation of the thermolysin digestion of the 110-kD (domain 4 without the ED-B sequence) and 120-kD (domain 4 containing the ED-B sequence) fragments. The *S*-cyanilation cleavage of the 85-kD fragment obtained from the 120-kD domain 4 is also schematically represented. The epitopes reacting with the mAbs 3E3, IST-4, and BC-1 are indicated. The numbers on the left (*1–6*) indicate the corresponding lanes of gels and immunoblots shown in Fig. 2 *B*. (*B*) On the left, a 4–18% SDS-PAGE gradient of the purified 110-kD domain 4 (lane *1*) and 120-kD domain 4 (lane *2*); thermolysin digest of the 110-kD fragment (lane *3*); thermolysin digest of the 120-kD fragment (lane *4*); purified 85-kD fragment from the thermolysin digestion of the 120-kD fragment (lane *5*) and the 85-kD fragment cleaved by *S*-cyanilation. The values on the left indicate the molecular masses, in kD, of the standards. The values on the right indicate the molecular masses, in kD, of the various FN fragments. On the right immunoblots of similar gels using the mAbs BC-1, IST-4, and 3E3, respectively, are shown.

BC-1, and a 35-kD fragment (Fig. 2) (44). The 85-kD fragment was purified and its carboxyl-terminal part containing the ED-B sequence was cleaved out by *S*-cyanilation. This generated a 65-kD fragment which no longer reacted with the mAb BC-1 (Fig. 2), demonstrating that the epitope recognized by this mAb is within the FN fragment containing the ED-B sequence which was removed.

To confirm the hypothesis that the mAb BC-1 recognizes an epitope within the ED-B sequence we used β-galactosidase–FN fusion proteins containing or not containing the ED-B sequence. A WI-38VA13 library in the expression vector λgt 11 was screened by an immunoenzymatic procedure using the mAb BC-1. All the positive clones obtained contained a FN DNA insert with at least part of the ED-B sequence as demonstrated by DNA sequencing. In particular, the clone λF2 produces a β-galactosidase–FN fusion protein in which the FN part is composed of the complete ED-B sequence plus 105 amino acids at its amino-terminal and 85 amino acids at its carboxyl-terminal ends (see Fig. 5). As a negative control we constructed a clone, λF6c, (see Materials and Methods) expressing a β-galactosidase–FN fusion protein identical to that produced by the cloned λF2 except that it lacked the entire ED-B sequence (Fig. 3). As shown in Fig. 3, the mAb BC-1 reacts only with the fusion protein containing the ED-B sequence from the clone λF2, but does not react with the fusion protein without the ED-B sequence produced by the clone λF6c. This demonstrates that the epitope recognized by the mAb BC-1 is localized within the ED-B sequence.

Fig. 4 shows a dot-blot analysis of plasma FN, FNs from three different normal human fibroblast cell lines and four transformed or tumor-derived cell lines using the mAbs IST-4 (which recognizes all different FN isoforms) and BC-1 (which recognizes only the B-FN isoform). While the mAb IST-4 reacts in the same way with all different FNs, the mAb BC-1 shows a reaction only with FNs from the SV-40-transformed human fibroblasts WI-38VA13 and with the rhabdomyosarcoma cell line RD.

Distribution of the B-FN Isoform in Human Fetal, Adult, and Tumor Tissues

Table I summarizes the results of the immunohistochemical analysis of a variety of fetal and normal adult tissues using the mAbs BC-1, which recognizes only the ED-B-containing FN molecules, B-FN, and IST-4, which recognizes all different FN isoforms. In adult tissues, while total FN has a widespread distribution (See Table I and reference 37), the presence of the B-FN isoform is limited to the superficial synovial cells, the intima of some ovarian vessels, scattered areas of the ovarian interstitium, isolated areas of the basement membranes of the celomic epithelium, and to areas of the myometrium (Table I). On the contrary, in tissues from 8–12-wk old fetuses the B-FN isoform is much more represented. In fact, among the tissues tested, the only negative ones were kidney, liver, colon, and skin tissues (see Table II). In particular the B-FN isoform is present in the intima of the vessels of fetal brain cortex, stomach, jejunum, thymus, and lung. The mAb BC-1 also stains some areas of the submucosa of the stomach and jejunum, and the basement membranes of the stomach and of developing bronchia (Fig. 5). However, in tissues from older fetuses (22–26 wk old) only the basal portion of the gastric and duodenal glands were found positive. This suggests that the B-FN isoform undergoes a programmed expression during ontogenesis.

Table III summarizes the results of the immunohistochemical analysis of 165 different primary human tumors using the mAbs IST-4 and BC-1. About 38% of the tumors tested showed the presence of the B-FN isoform (Table III and Fig. 5). Within a given tumor type, the incidence of tumors showing the presence of the B-FN isoform was variable and no correlation was found with the degree of differentiation (colon adenocarcinomas) or the tumor histotype (breast tumors).

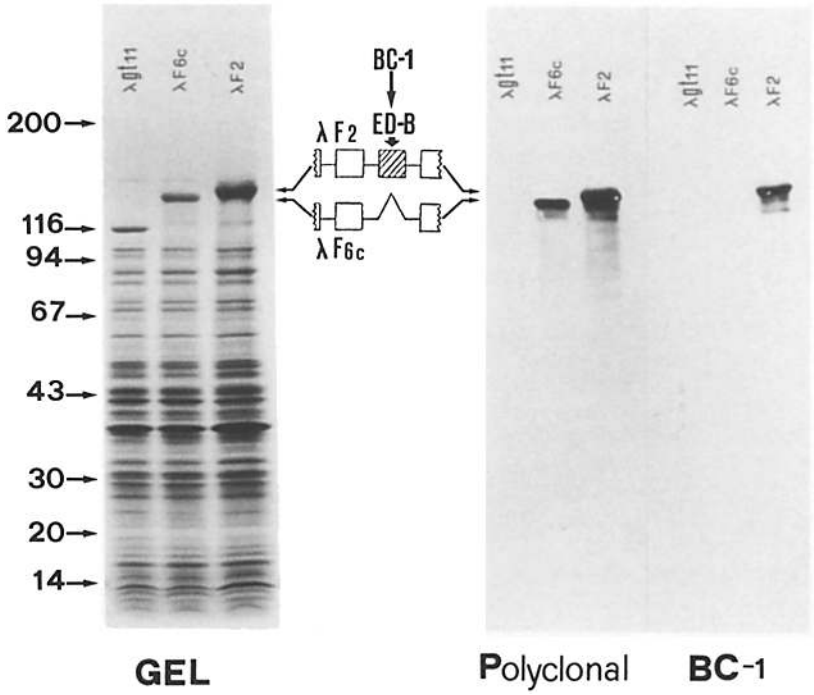

Figure 3. On the left, a 4–18% SDS-PAGE gradient of proteins from *E. coli* infected by the expression vector λgt 11 and by the clones λF2 and λF6c, respectively. The clones λF2 and λF6c produce the fusion proteins depicted in the middle of the figure. On the right, immunoblots using the mAb BC-1 and a polyclonal rabbit antiserum to human FN. The values on the left indicate the molecular masses, in kD, of the standards.

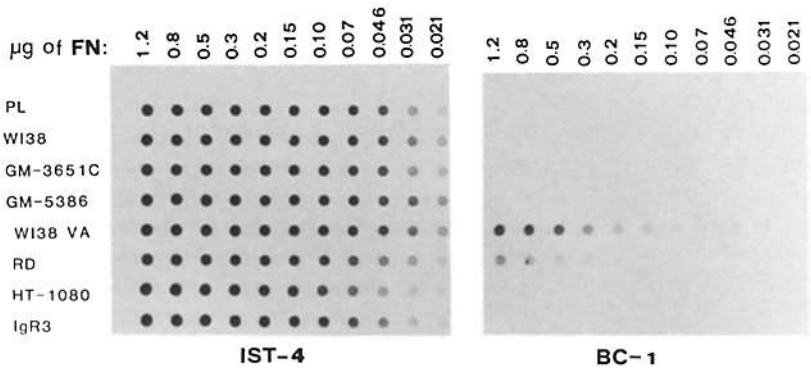

Figure 4. Dot-blot analysis of plasma FN (*PL*), and FNs from the following human cell lines: WI-38, embryonic lung fibroblasts; GM-3651-G, adult skin fibroblast; GM-5386, fetal skin fibroblasts; WI-38VA13, SV-40-transformed embryonic lung fibroblasts; *RD*, from a rhabdomyosarcoma; HT-1080, from a fibrosarcoma; and IgR3, from a melanoma. The mAb IST-4 (*left*) recognizes all different FN isoforms while the mAb BC-1 (*right*) recognizes only the B-FN isoform.

The only exceptions were meningioma of psammomatous and meningotheliomatous type which were found to be consistently stained by the mAb BC-1. In some cases of renal, pulmonary, and colonic carcinomas, in which both normal and tumor tissues of the same patients could be studied, the B-FN isoform was found only in the transformed tissue. In almost all the specimens the B-FN isoform was confined to areas of variable extension of the tumor interstitium which surrounded or divided tumor cell nests of variable size. Staining was seldom found to outline scattered individual cells. A frequent feature was the presence of the B-FN isoform in the vascular intima which was never present in normal adult tissues with the exception of some ovarian vessels.

We also tested, with the mAb BC-1, three cutaneous scars (10–30 d old), and different benign and chronic inflammatory lesions: four breast fibroadenoma and one adenofibroma, five prostate hyperplasia, two fibrocystic disease samples, four gynecomastia, four urinary bladder and colorectal polyps, 14 intradermal nevus, two angioma, two ovarian cystoadenoma, two thyroid adenoma, one neurofibroma, two liver cirrhosis samples, and two hydatidosis cysts. All of them were found negative with the exception of the breast adenofibroma, which was variably positive in some interstitial areas.

Table I. Reactivity of Normal Adult Tissues with mAbs BC-1 and IST-4

	BC-1	IST-4
Brain (2)	−	+
Lung (3)	−	+
Breast (3)	−	+
Stomach (4)	−	+
Duodenum (3)	−	+
Colon (3)	−	+
Liver (2)	−	+
Pancreas (1)	−	+
Kidney (4)	−	+
Urinary bladder (2)	−	+
Prostate (2)	−	+
Testis (2)	−	+
Endometrium (2)	−	+
Spleen (2)	−	+
Lymphnode (2)	−	+
Skin (6)	−	+
Thymus (2)	−	+
Thyroid (2)	−	+
Meninges (2)	−	+
Skeletal muscle (1)	−	+
Choroid (2)	−	+
Retina (2)	−	+
Synovia (2)	+	+
Ovary (2)	+	+
Fallopian tubes (4)	−	+
Myocardium (1)	−	+
Choroid plexus (1)	−	+
Placenta (2)	−	+
Myometrium (4)	+	+
Celomic epithelium (2)	+	+

The number of individuals from which the tissues were obtained is indicated in parenthesis. The mAb BC-1 is specific only for the B-FN isoform, while the mAb IST-4 recognizes all different FN isoforms.

Discussion

In contrast to FN from normal human fibroblasts, in which the ED-B-containing molecules (B-FN) are barely detectable, in FN from SV-40-transformed cells the B-FN isoform represents ~30–40% of the molecules. This is due to a different splicing pattern of FN pre-mRNA which leads, in transformed cells, to high level expression of the exon ED-B (44).

In this paper we report on the production and characterization of an mAb (BC-1) which recognizes an epitope within the ED-B sequence. This localization has been established on the basis of the following results. (*a*) In immunoblotting experiments, the mAb BC-1 does not react with plasma FN; it shows a barely detectable reaction with FN from normal

Table II. Reactivity of Fetal (8–10-wk) Tissues with mAbs BC-1 and IST-4

	BC-1	IST-4
Brain cortex	+	+
Liver	−	+
Colon	−	+
Skin	−	+
Kidney	−	+
Stomach	+	+
Thymus	+	+
Lung	+	+
Jejunum	+	+

The mAb BC-1 is specific only for the B-FN isoform while the mAb IST-4 recognizes all different FN isoforms. In the same tissues and in those of the duodenum, spleen, pancreas, and urinary bladder of a 26-wk-old fetus, only the stomach and duodenum were found positive with the mAb BC-1.

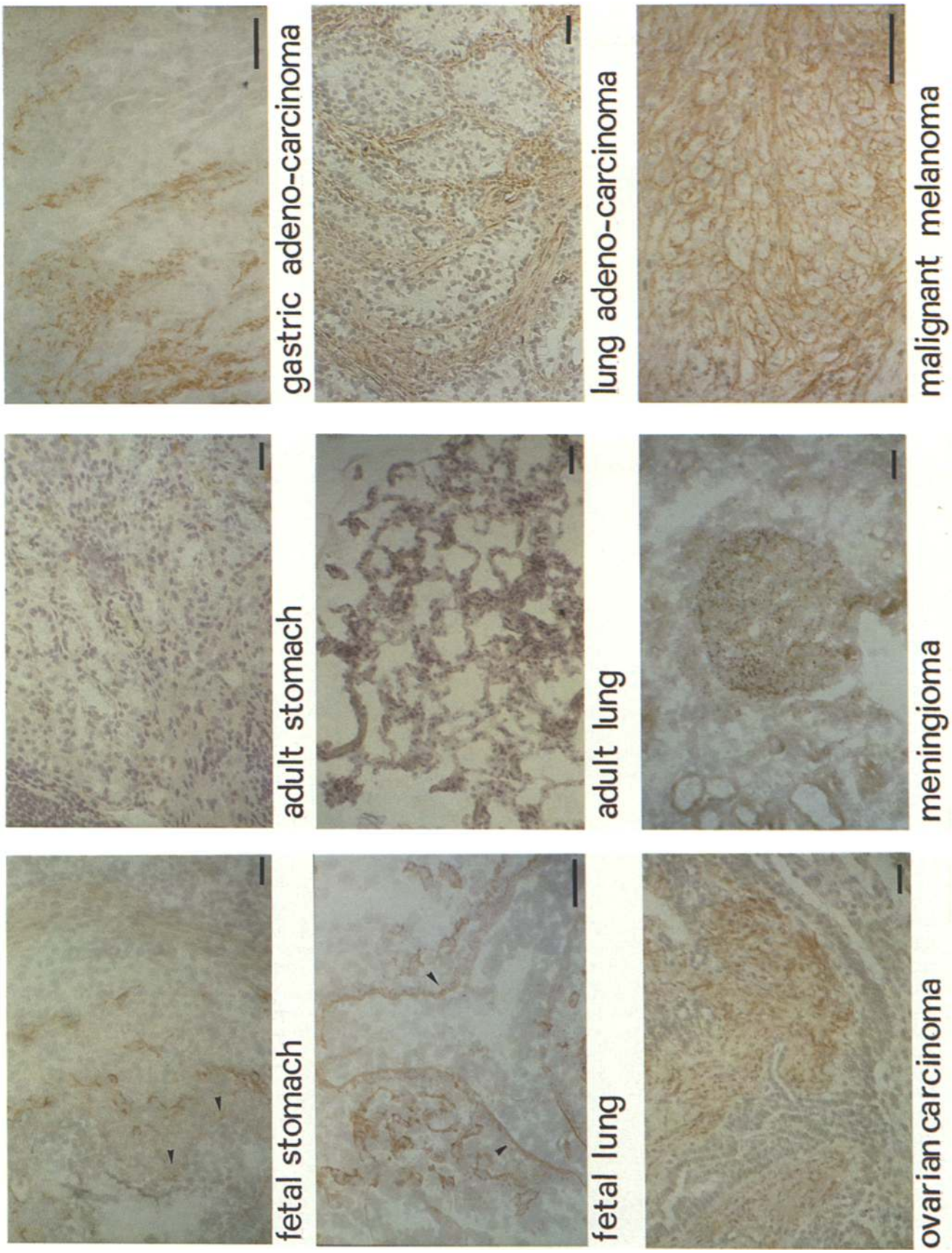

Figure 5. Study of the distribution of the B-FN isoform in human fetal, adult, and tumor tissues by indirect immunoperoxidase staining using the mAb BC-1. The mAb BC-1 does not react with adult stomach but shows a positive reaction along the basement membranes, (*arrowheads*) and in the wall of the submucosal vessels of the fetal stomach and in the septa of the gastric carcinoma. The mAb BC-1 does not react with the adult lung parenchyma but reacts with the basement membrane of the developing bronchia (*arrowheads*) and the wall of the submucosal capillaries of the fetal lung and the septa of the lung carcinoma. The mAb BC-1 shows positive reaction in the interstitium of the ovarian carcinoma, a punctuated pattern of staining with meningioma and outlines the boundaries of the cells in malignant melanoma. All these tissues showed a strong positive reaction when tested using the mAb IST-4 which recognizes all different FN isoforms. Bar, 10 μm.

Table III. Reactivity of the mAb BC-1 with Primary Tumors of Various Histotypes

Tumor type	Number positive/ Number tested
Stomach adenocarcinoma	3/7
Pancreas adenocarcinoma	0/3
Liver adenocarcinoma	2/7
Colon adenocarcinoma	2/10
Kidney clear cell carcinoma	4/8
Urinary bladder carcinoma	4/8
Prostate adenocarcinoma	1/5
Ovary adenocarcinoma	2/8
Endometrium adenocarcinoma	1/6
Skin carcinoma (mixed histotypes)	3/8
Thyroid adenocarcinoma	1/8
Lung carcinoma (mixed histotypes)	7/13
Breast adenocarcinoma	3/13
Skin and ocular melanoma	9/20
Brain tumor (mixed histotype)	4/13
Brain meningiomas	13/13
Sarcomas (mixed histotypes)	3/15
Total	62/165

All tumors tested were strongly positive with mAb IST-4 which recognizes all different FN isoforms. The mAb BC-1 recognizes only the B-FN isoform.

human fibroblasts but shows a very strong reaction with FN from SV-40-transformed fibroblasts (Fig. 1). These results are in agreement with those previously reported showing the absence of B-FN isoform in plasma, a very small amount in FN from normal fibroblasts, and a large amount in FN from SV-40-transformed fibroblasts (44). (*b*) Immunoblotting experiments, using different FN fragments, demonstrated that the mAb BC-1 reacts with an epitope localized in the central part of the 120-kD domain 4 (which contains the ED-B sequence) but does not react with the same domain lacking the ED-B sequence (110 kD) (Fig. 2). (*c*) The mAb BC-1 reacts with the fusion protein λF2 which contains the ED-B sequence, but does not react with the fusion protein λF6c which is identical to λF2 except that it lacks the ED-B sequence (Fig. 3); this also demonstrates that the mAb BC-1 recognizes a primary sequence epitope.

Previously, Matsuura and Hakomori (25), using the mAb FDC-6, suggested the existence of a domain present in FN from tumor and fetal tissues, but absent in FN from normal adult tissues. We exclude the possibility that the mAb FDC-6 recognizes an antigenic determinant within the ED-B sequence since the authors have localized this epitope within the IIICS sequence (see Fig. 1). Furthermore, the mAb FDC-6 does not recognize a primary sequence epitope (26).

Using the mAb BC-1 we compared the amount of the B-FN isoform in a panel of FNs from normal, tumor-derived, and transformed human cell lines using the dot-blot technique. The results demonstrate that the B-FN molecules are clearly detectable only in FNs from the SV-40-transformed, WI-38VA13 cell line and from the rhabdomyosarcoma cell line RD. Furthermore, the use of a more sensitive technique demonstrated that all the transformed cell lines tested produce FN in which the percentage of molecules containing the ED-B sequence is higher than in FN from normal human fibroblasts (Zardi, L., L. Bossi, and B. Carnemolla, manuscript in preparation). This increase of B-FN in transformed cells, with respect to normal cells, observed using the mAb BC-1 is in agreement with the data previously reported both at the mRNA and at the protein level (44). However, Schwarzbauer et al. (33) and Norton and Hynes (28) did not observe any significant increase in the relative amount of ED-B containing FN mRNA in transformed embryonic rat and chicken cells compared to the normal counterpart. At this moment, we cannot explain the differences between the above mentioned data and ours. Analysis, both at the protein and mRNA level, of larger panels of cultured cells from different species may be useful in clarifying these discrepancies. Furthermore, Castellani et al. (8) and Borsi et al. (5) have demonstrated that FNs from transformed or tumor-derived cells are composed of a population of molecules in which both the IIICS and ED-A sequences are expressed more than in FN from normal cells. The reason why tumor cells tend to express more of the FN sequences regulated by alternative splicing mechanisms has yet to be established.

The observed increased expression of the B-FN isoform in transformed, compared to normal, cell lines prompted us to study the distribution of the B-FN isoform in primary tumors of various histotypes and in normal adult and fetal tissues. In contrast to the widespread distribution of other FN isoforms, only a few normal adult tissues showed the presence of the B-FN isoform (see Table I). Unlike normal adult tissues, 38% of the 165 tumors tested displayed considerable levels of the B-FN isoform. It was distributed in the interstitium which surrounded the tumor cells, and in the vascular intima which in normal tissues was only detected in some ovarian vessels. The incidence of positive tumors was variable within type, with the exception of meningioma in which 13 cases out of 13 showed a positive reaction with the mAb BC-1. We have been unable to find any correlation between the presence of B-FN and the degree of tumor differentiation or histotype. This leaves the question open as to why some tumors show the presence of B-FN and others do not. Another unanswered question is whether the B-FN isoform is produced by the tumor cells or by the mesenchymal cells which surround the tumors. One possibility is that the tumor cells release factor(s) which stimulate the mesenchymal cells to synthesize the B-FN isoform. On the other hand, B-FN was absent from all the benign lesions, chronic inflammatory lesions, and cutaneous scars tested, with the single exception of a breast adenofibroma which displayed staining in only a few areas of the interstitium. In tissues from 8–10-wk-old fetuses, the B-FN isoform is more highly represented compared to adult tissues, but in those from 26-wk-old fetuses only the stomach and duodenum were found positive. These data are in agreement with those of Norton and Hynes (28) showing that B-FN mRNA was the predominant form in total embryo RNA from 2.5 to 11 d after fertilization.

These results clearly show a switching in the accumulation of the different FN isoforms associated with human ontogenesis and the reverse process associated with oncogenesis. The presence of the B-FN isoform in fetal tissues and its reappearance in tumors suggests that B-FN may play a role common to both situations and strongly indicates that transformation alters the splicing pattern of FN RNA towards that characteristic of fetuses.

Alternative RNA splicing is an important and widespread mechanism of gene regulation. The differential expression of exons into mature mRNAs is often under developmental

and/or tissue-specific regulation. It has been suggested that alternative splicing may be regulated by information encoded in the gene transcript (*cis*) but may require diffusible factor(s) (*trans*) that may be responsible for the developmental and/or tissue-specific regulation of splicing (3, 4, 13, 23, 30). Since transformation is associated with a deep modification of gene expression, it is not surprising that such a fine mechanism of gene regulation, as RNA splicing, may be altered in transformation. The alternative splicing of messenger RNA precursors in tumor cells and the biological function(s) of the sequences which are variably expressed may be fertile future research areas.

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