Journal of Cell Science 104, 783-792 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Fibronectin splice variants are differentially incorporated into the extracellular matrix of tumorigenic and non-tumorigenic hybrids between normal fibroblasts and sarcoma cells

Helen J. Mardon*,†, Richard P. Grant, Kate E. Grant† and Henry Harris

Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, UK

*Author for correspondence
†Present address: Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

SUMMARY

Recent reports have described transformation- and tumour-specific expression of fibronectin isoforms generated by alternative splicing of the fibronectin pre-mRNA. We have investigated the expression and distribution of EDIIIA+ and EDIIIB+ fibronectin splice variants in tumorigenic and non-tumorigenic somatic cell hybrids made by fusing fibrosarcoma-derived cells (HT1080) and normal fibroblasts (GM00097). Alternative splicing of EDIIIA and EDIIIB was assessed quantitatively by S_1 nuclease analyses. The levels of EDIIIA+ and EDIIIB+ fibronectin mRNAs were similar in the parental and hybrid cells. Domain-specific monoclonal antibodies were used in immunohistochemical studies to identify EDIIIA+ and EDIIIB+ fibronectins in fixed cells. GM00097 and the non-tumorigenic hybrid (clone G3) showed high levels of both EDIIIA+ and EDIIIB+ fibronectin staining. The tumorigenic hybrid (clone C1) showed reduced amounts of EDIIIA+ fibronectin, but no detectable EDIIIB+ fibronectin. No fibronectin was detected on the surface of HT1080 cells. Western blots of protein extracted from culture supernatants and extracellular matrices revealed that GM00097 and G3 cells incorporated most of the EDIIIA+ and EDIIIB+ fibronectin into the extracellular matrix whereas C1 cells released a large proportion of the EDIIIA+ fibronectin, and almost all of the EDIIIB+ fibronectin, into the supernatant. We conclude that there are differences in the presence of EDIIIA+ and EDIIIB+ FNs on the surface of tumorigenic and non-tumorigenic cells and that these differences are due to differential incorporation of FN variants into the ECM.

Key words: alternative splicing, extracellular matrix, fibronectin, somatic cell hybrids, tumorigenesis

INTRODUCTION

The striking correlation between oncogenic transformation and the loss of fibronectins (FNs) from the cell surface was first described in 1973 (Hynes, 1973). It was subsequently discovered that addition of plasma fibronectin to cultures of transformed fibroblasts restores to the cell a normal phenotype (Yamada et al., 1976). These observations initiated a field of research into the role of FNs in transformation and malignancy (reviewed by Hynes, 1990). Evidence for a correlation between loss of cell-surface FN and tumorigenicity is less convincing and is complicated by differences in the expression of FNs by tumorigenic cells *in vitro* and tumours *in vivo*. Furthermore, transformation and tumorigenesis are different processes, and changes in FN expression that occur with transformation do not necessarily occur in tumours. Nevertheless, a good correlation between loss of FNs and tumorigenicity has been shown for some transformed cell lines (Chen et al., 1976; Gallimore et al., 1977).

The complexity of the roles of FNs in biological processes such as tumorigenesis is augmented by the existence of up to twenty FN isoforms. These show structural diversity but contain a series of the repeating amino acid sequences: type I, type II and type III repeats (reviewed by Hynes, 1985; Owens et al., 1986; see Fig. 1). FN isoforms are generated by alternative splicing of a single pre-mRNA in three regions called EDIIIA, EDIIIB and IIICS in man (Kornblihtt et al., 1984a,b; Zardi et al., 1987), and EIIIA, EIIIB and V in rat (Schwarzbauer et al., 1983), chicken (Norton and Hynes, 1987) and *Xenopus* (DeSimone et al., 1992). EDIIIA and EDIIIB each encode one complete type III repeat, which can be either included in, or excluded from, the FN molecule as a result of alternative splicing (Fig. 1). The IIICS region encodes two cell type-specific binding sites, CS1 and CS5 (Humphries et al., 1987) the expression of which is determined by a more complex pattern of alternative splicing (Mardon and Sebastio, 1992). Differences in the splicing patterns of EDIIIA, EDIIIB and IIICS account for the structural diversity of plasma and cel-

lular FNs (Schwarzbauer et al., 1983; Kornblihtt et al., 1984a,b).

A number of normal, transformed and tumorigenic cells and embryonic, normal adult, and malignant tissues have been analysed for differential expression of EDIIIA+ and EDIIIB+ mRNAs (Kornblihtt et al., 1984a,b; Norton and Hynes, 1987; Zardi et al., 1987; Oyama et al., 1989, 1990), and EDIIIA+ and EDIIIB+ FNs by the use of domain-specific monoclonal antibodies (Sekiguchi et al., 1985; Borsi et al., 1987; Vartio et al., 1987; Carnemolla et al., 1989). The results of these and other studies suggest that expression of EDIIIB+ FN is elevated during development and in some transformed and tumour cells relative to their normal adult counterparts in which EDIIIA+ FN is predominant.

Studies in which direct comparisons may be made between cells which differ in tumorigenicity, but which are isogenic in the sense that they are derivatives of the same parental cells, are particularly useful for examining the relationship between FNs and tumorigenicity. Somatic cell hybrids derived from one normal and one tumorigenic parent cell provide an excellent cell system for this kind of study. These hybrids, which may either be tumorigenic or suppressed with respect to tumorigenicity, have been used extensively in the genetic analysis of tumorigenesis (reviewed by Harris, 1988, 1990). Steel and Harris (1989) demonstrated that transfection of antisense FN cDNA into fibroblast × melanoma hybrids in which tumorigenicity was suppressed inhibited FN expression and restored the ability of these cells to make tumours in nude mice. However, the correlation between tumorigenesis and loss of FN was apparent in some hybrid cells (e.g. see Der et al., 1981; Zajchowski et al., 1990) but not in others (Der and Stanbridge, 1978).

In this study we address the possible relationship between FN variants and tumorigenicity (as opposed to transformation). We have used tumorigenic and non-tumorigenic somatic cell hybrids made by fusing normal fibroblasts and fibrosarcoma cells to examine more closely the relationship between tumorigenesis and expression of EDIIIA+ and EDIIIB+ FNs. We provide measurements of the differential expression of EDIIIA+ and EDIIIB+ FN mRNA and protein in the parental cells, a hybrid clone in which tumorigenicity is suppressed and a tumorigenic hybrid, and we describe differences in the destination of FN variants in cultures of these cells.

MATERIALS AND METHODS

Cell culture

GM00097 human diploid fibroblasts (Punnett et al., 1974) were maintained in MEM (GIBCO, UK) + 20% foetal calf serum (FCS; GIBCO, UK). A human fibrosarcoma cell line HT1080 (Rasheed et al., 1974), which is HPRT-deficient, and the hybrid clones C1 and G3, were maintained in DMEM + 10% FCS. Cultures were grown to subconfluence either on pre-washed, no. 2, 22 mm coverslips (Chance, UK), or in 80 cm^2 tissue culture flasks (Flow, UK) and then maintained for a further 24 h in serum-free medium containing 1:1000 (w:v) insulin-transferrin-sodium selenite supplement (Boeringher Mannheim UK). Cultures were processed for RNA and protein assays as described below. All the experiments were done on cultures between the following passage numbers: GM00097, 9-16; HT1080, 26-36; clone C1, 39-50; clone G3, 10-31. Cell cultures were photographed using a Leitz Diavert microscope.

Cell fusion

A total of 10^5 each of GM00097 and HT1080 cells were fused by means of Sendai virus as described by Harris and Watkins (1965). Hybrid clones were selected in medium containing HAT (Littlefield, 1964), expanded in culture, and assayed for tumorigenicity in nude mice. Chromosome preparations were made as described by Evans et al. (1982).

Assay for tumorigenicity

Suspensions of 10^6 C1 or G3 cells in 100 µl DMEM were injected into the flanks of nude mice. At least 9 mice were injected with cells from each clone, in batches of 3 from different passage numbers within those stated above. The mice were inspected daily for the appearance of tumours, for up to 92 days.

S_1 nuclease protection assay

RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction of cells (Chomczynski and Sacchi, 1987). Probes were prepared by labelling the *Bst*EII/*Eco*RI fragment from pGEM/A+1 (for EDIIIA probes), and the *Bam*HI/*Ssp*I fragment from pGEM/B− (for EDIIIB probes), with [γ-^{32}P]ATP using bacteriophage T4 polynucleotide kinase. pGEM/A+1 and pGEM/B− were constructed by cloning the 289 bp *Ava*I/*Ava*II fragment pGEM/A+1 and the 475 bp *Bam*HI/*Sac*I fragment (pGEM/B−) into the *Hin*cII site of pGEM/3Z. S_1 nuclease protection assays were performed as described by Proudfoot et al. (1984). RNA-DNA hybrids were fractionated by 7 M urea/polyacrylamide gel electrophoresis (PAGE). Gels were exposed to X-ray film for 24 h at −70ºC. Densitometric analysis of the autoradiographs was done with a LKB Ultrascan densitometer.

Cytochemical and immunohistochemical localisation of protein

Cells grown on coverslips were fixed in 95% ethanol for 5 min at 0ºC, and air-dried. Actin was visualised by incubation with 3 units/ml rhodamine phalloidin (Molecular Probes, USA), according to the manufacturer's instructions. FN variant-specific mAbs IST-4, IST-9 and BC-1 (Sekiguchi et al., 1985; Borsi et al., 1987; Carnemolla et al., 1989), kindly given to us by L. Zardi, were used to localise all FNs, EDIIIA+ FN and EDIIIB+ FN, respectively. Immunohistochemical procedures have been described elsewhere (Mardon et al., 1987). Labelled cells were viewed and photographed with a Zeiss Axioplan microscope.

Protein extraction and Western blot analyses

Protein was precipitated from culture supernatants by the addition of 2-3 volumes of ice-cold 95% ethanol. The precipitated protein from individual replicate 80 cm^2 flasks was collected by centrifugation at 2000 revs/min for 5 min and resuspended in 100 µl protein sample buffer (Laemmli, 1970). Cells were lysed with 0.02 M NH_4OH (Gospodarwicz et al., 1984) and the lysate collected. The material remaining attached to the flask was washed extensively with PBS, and resuspended in 400 µl protein lysis buffer (1% Triton X-100; 0.5% sodium deoxycholate; 0.5% SDS; 0.1% NaCl; 5 mM $MgCl_2$; 50 mM Tris-HCl, pH 7.6) containing 3 mM phenylmethylsulfonyl fluoride, and 1 µg/ml (w/v) each of aprotinin, leupeptins and pepstatin. Proteins were precipitated and collected as above and resuspended in 100 µl protein sample buffer, and a sample was electrophoresed through a 4% polyacrylamide stacking gel and 6% polyacrylamide resolving gel containing 0.1% SDS (Laemmli, 1970). Proteins were transferred onto nitrocellu-

lose filters (Towbin et al., 1979). Actin, the 60 kDa actin-related protein, and FN variants, were detected with the ECL Western Blotting System (Amersham), according to the manufacturer's instructions. Anti-actin mAbs (Sigma) were followed by mAbs IST-4; IST9 or BC-1 and then by sheep anti-mouse IgG conjugated to horseradish peroxidase (HRP; Sigma, UK). Prestained protein markers (Gibco BRL, UK) were lysozyme (15 kDa); β-lactoglobulin (18 kDa); carbonic anhydrase (28 kDa); ovalbumin (43 kDa); BSA (71 kDa); phosphorylase *b* (100 kDa) and myosin (H-chain) (218 kDa). Filters were exposed to X-ray film and densitometric analysis of images was done as above.

RESULTS

HT1080 × GM00097 hybrid clone G3 shows suppression of tumorigenicity whereas clone C1 is tumorigenic

The fusion of GM00097 fibroblasts and HT1080 fibrosarcoma cells gave rise to non-tumorigenic and tumorigenic hybrid cells whose hybrid nature was confirmed by chromosome analysis. The tumorigenicity of two such hybrid clones, C1 and G3, is shown in Table 1. Clone C1 was highly tumorigenic when inoculated into nude mice. Tumours appeared in all 11 mice injected with 10^6 C1 cells after a short latency (3-12 days) and grew rapidly (>1.5 cm diameter after 16 days), whereas clone G3 cells produced a tumour in only 1 out of 9 mice injected. Furthermore, this tumour had a long latency (44 days), and was small (<0.25 cm diameter) even after 92 days.

The tumorigenic and non-tumorigenic hybrids are morphologically different

The morphologies of the two parent cell lines, GM00097 and HT1080, and the hybrid clones C1 and G3, viewed by phase-contrast microscopy, are shown in Fig. 2. GM00097 cells are large, flat and exhibit typical fibroblastic morphology. HT1080 cells are small and rounded, and their morphology is characteristic of that of a transformed cell. G3 cells are relatively large and, like GM00097 cells, exhibit a high degree of spreading, whereas C1 cells are smaller and more rounded and resemble HT1080 cells.

Actin organisation is disrupted in HT1080 and C1 cells

Localisation of F-actin by rhodamine-phalloidin (Fig. 3) shows that GM00097 fibroblasts have a well-organised cytoskeleton. Actin is localised in prominent stress fibres, which are features of many non-transformed cells in culture (Burridge et al., 1988). In HT1080 cells the actin staining is bright, but diffuse. The actin is localised in small patches, rather than in linear stress fibres and in a pericellular rim. G3 cells have a pattern of actin staining which is similar to the GM00097 parent cells, and stress fibres are clearly visible in most of the cells. The pattern of actin staining in C1 cells is similar to that observed for HT1080 cells and to that described by Carley et al. (1981) for transformed cells. Actin is very diffuse across the cell and stress fibres are not visible. A pericellular rim and small, diffuse patches of actin, can be clearly identified.

Alternative splicing of EDIIIA and EDIIIB in the hybrids is not related to tumorigenicity

The pattern of splicing of FN pre-mRNA in the EDIIIA and EDIIIB regions was investigated by S_1 nuclease analysis of RNA from GM00097, HT1080, and clones C1 and G3 (Fig. 4). The amounts of EDIIIA+ and EDIIIB+ mRNA detected by densitometric analyses of data from three independent experiments were expressed as a percentage of the total FN mRNA. Different amounts of RNA were used from each cell type to facilitate the analysis of a single exposure of the autoradiographs. Taking into account these loading differences, comparison of the intensity of the bands corresponding to the total amount of FN mRNA (i.e. EDIIIA+ plus EDIIIA− in Fig. 4A, and EDIIIB+ plus

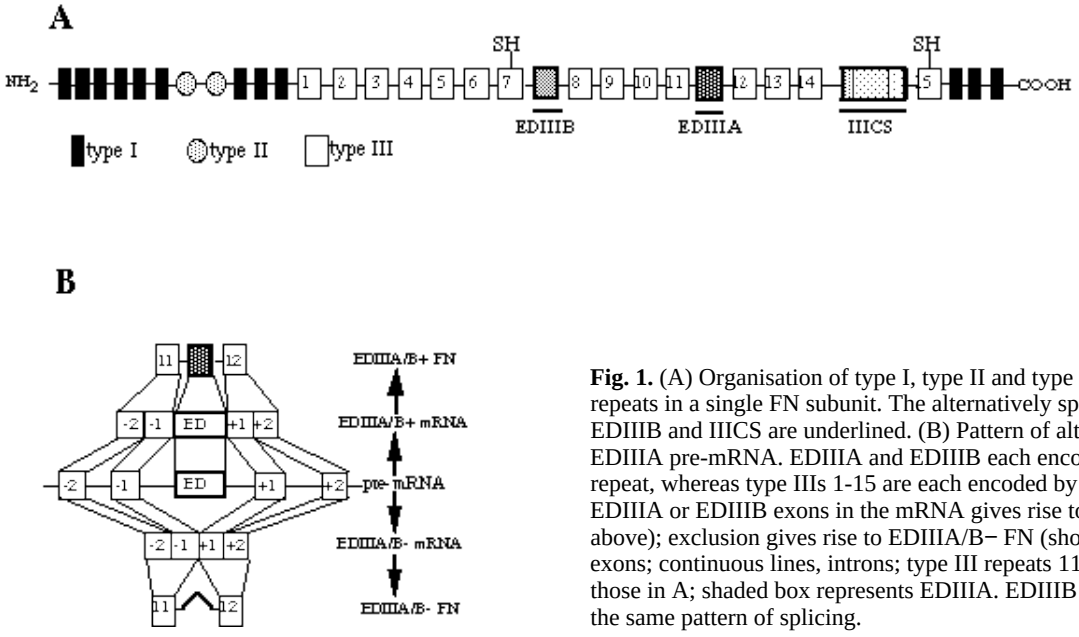

Fig. 1. (A) Organisation of type I, type II and type III (1-15) amino acid repeats in a single FN subunit. The alternatively spliced regions EDIIIA, EDIIIB and IIICS are underlined. (B) Pattern of alternative splicing of EDIIIA pre-mRNA. EDIIIA and EDIIIB each encode a single type III repeat, whereas type IIIs 1-15 are each encoded by two exons. Inclusion of EDIIIA or EDIIIB exons in the mRNA gives rise to EDIIIA/B+ FN (shown above); exclusion gives rise to EDIIIA/B− FN (shown below). Open boxes, exons; continuous lines, introns; type III repeats 11 and 12 correspond to those in A; shaded box represents EDIIIA. EDIIIB pre-mRNA undergoes the same pattern of splicing.

Table 1. Tumorigenicity of GM00097 × HT1080 hybrid cells injected into nude mice

Hybrid	No. cells injected	No. mice with tumours / no. mice injected	Latency (days)	Size of tumour (diameter)
Clone C1	10^6	11/11	3-12	≥1.5 cm at 16 days
Clone G3	10^6	1/9	44	≤0.25 cm at 92 days

EDIIIB− in Fig. 4B) shows that GM00097 cells expressed relatively high levels and HT1080 cells very low levels of FN. However, the level of FN expression in clones C1 and G3 was similar. Both cell lines expressed higher levels of FN mRNA than the HT1080 cells (at least 10-fold more), but less than GM00097 cells.

The S_1 nuclease analysis in Fig. 4A shows, that compared to HT1080, C1 or G3 cells, GM00097 cells expressed a higher proportion of EDIIIA− mRNA than HT1080, C1 or G3 cells. High levels of EDIIIA− FN mRNA have previously been reported in normal fibroblasts (Borsi et al., 1990). All four cell lines synthesized a lower proportion of EDIIIB+ mRNA than EDIIIB− mRNA (Fig. 4B). These data also show that all four cell types processed both EDIIIA and EDIIIB exons accurately. Densitometric analyses of the autoradiographs (Fig. 4C) confirmed that the percentages of EDIIIA+ and EDIIIB+ mRNA are similar in all four cell lines. GM00097 cells express 65% EDIIIA+ mRNA (range 61.5-67.6%) whereas HT1080 cells express 89% (range 86.1-94.5%), C1 cells 94% (range 92.2-97.4%) and G3 cells 86% (range 86.2-86.9%) EDIIIA+ mRNA. Nevertheless, the difference between GM00097 and C1 cells is less than 2-fold (or less than a 6-fold difference in the expression of EDIIIA− mRNA). The greatest difference in the expression of EDIIIB+ mRNA is between G3 and GM00097 and is less than 3-fold. These data are in agreement with previously reported levels of EDIIIB+ mRNA for HT1080 (Barone et al., 1989) and for some normal skin fibroblasts (Borsi et al., 1992). Our results suggest that there is no significant difference in the regulation of splicing of EDIIIA+ and EDIIIB+ pre-mRNA in GM00097, HT1080, or clones C1 and G3.

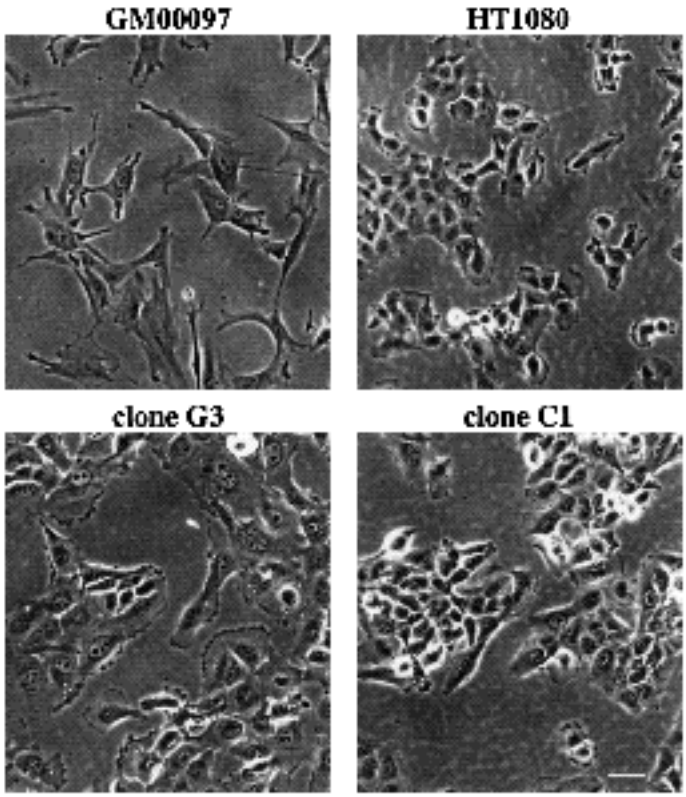

Fig. 2. Morphologies of GM00097, HT1080, C1 and G3 cells in culture observed by phase contrast microscopy. Bar, 10 μm.

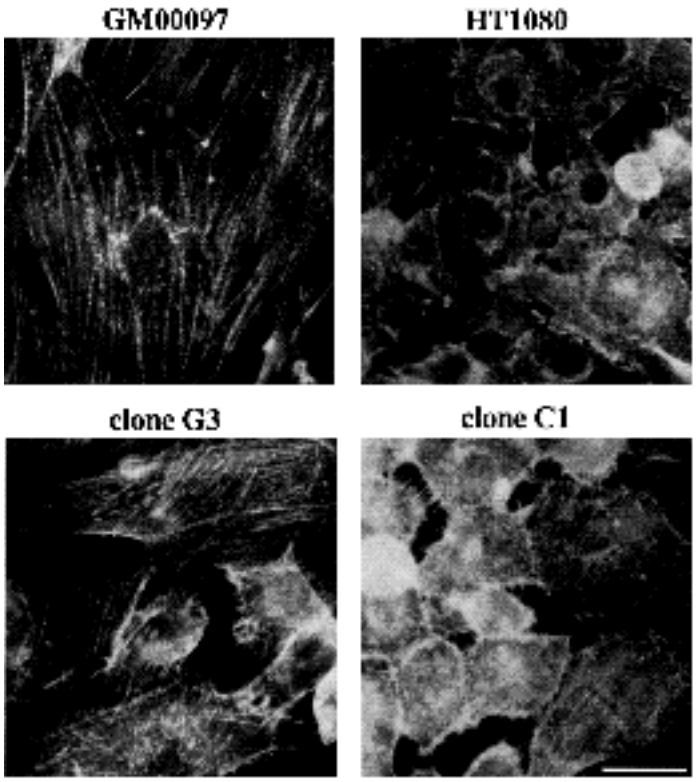

Fig. 3. Localisation of F-actin by rhodamine-phalloidin in fixed cultures of GM00097, HT1080, and clones C1 and G3. Bar, 10 μm.

EDIIIA+ but not EDIIIB+ FN is present in the ECM of the tumorigenic clone C1

Domain-specific mAbs were used to detect the presence of all forms of FN (IST-4), EDIIIA+ FNs (IST-9), and EDIIIB+ FNs (BC-1) in the cultures of the parental and hybrid cells (Fig. 5). The staining obtained with IST-4 shows that both GM00097 and G3 cells secrete a FN-rich ECM. No staining with IST-4 was observed in cultures of HT1080, indicating that FN was not present in the ECM of these cells. FNs were detected in the ECM of C1 cells, but at reduced levels compared to the ECM of GM00097 and G3 cells. The level of staining with IST-9 was similar to that observed for IST-4 for each cell type. EDIIIA is thus abundant in the matrix of GM00097 and G3 cells, less abundant in C1 cells, and, as would be expected from the results obtained with

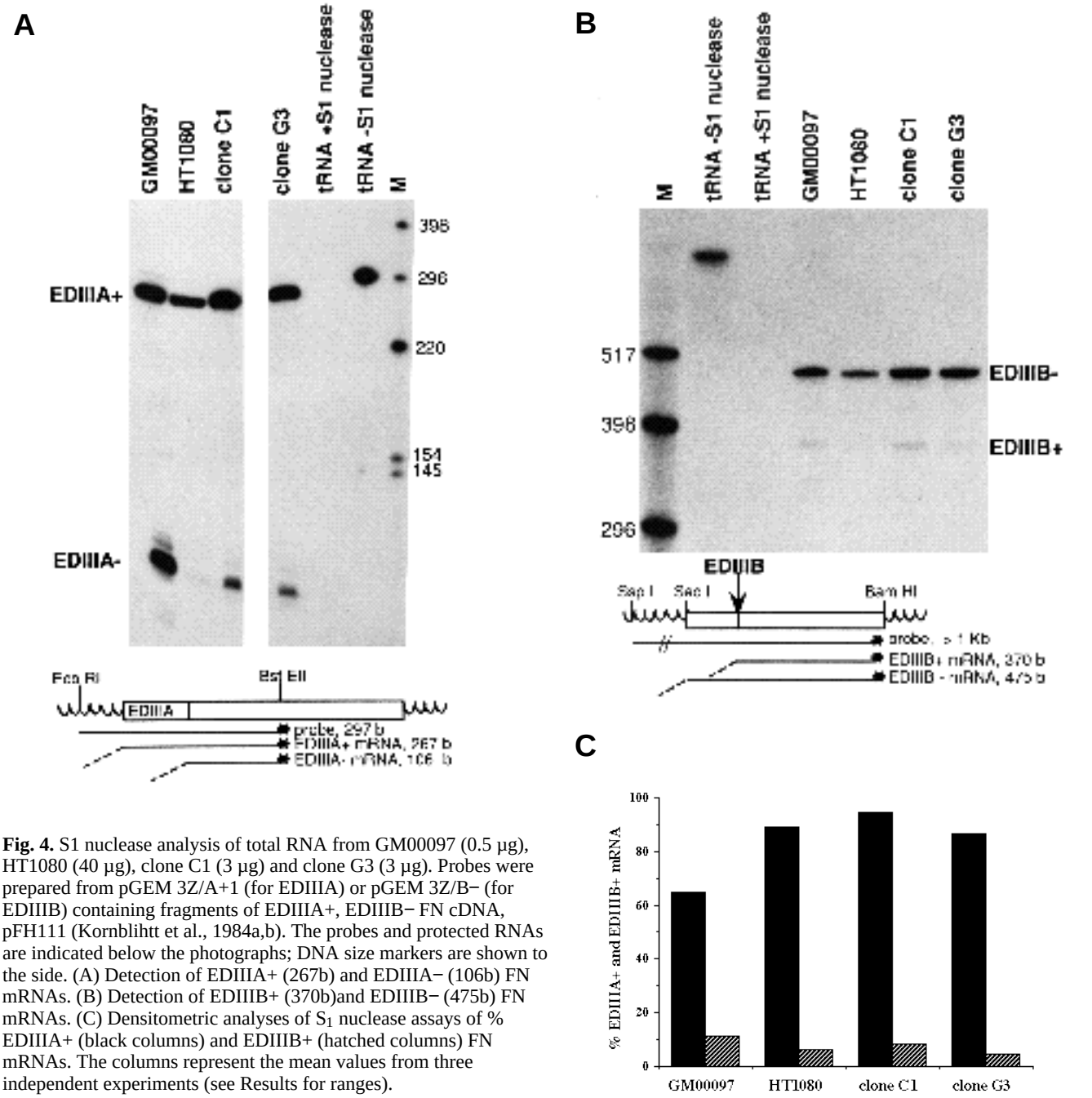

Fig. 4. S1 nuclease analysis of total RNA from GM00097 (0.5 μg), HT1080 (40 μg), clone C1 (3 μg) and clone G3 (3 μg). Probes were prepared from pGEM 3Z/A+1 (for EDIIIA) or pGEM 3Z/B− (for EDIIIB) containing fragments of EDIIIA+, EDIIIB− FN cDNA, pFH111 (Kornblihtt et al., 1984a,b). The probes and protected RNAs are indicated below the photographs; DNA size markers are shown to the side. (A) Detection of EDIIIA+ (267b) and EDIIIA− (106b) FN mRNAs. (B) Detection of EDIIIB+ (370b)and EDIIIB− (475b) FN mRNAs. (C) Densitometric analyses of S_1 nuclease assays of % EDIIIA+ (black columns) and EDIIIB+ (hatched columns) FN mRNAs. The columns represent the mean values from three independent experiments (see Results for ranges).

IST-4, absent in HT1080 cells. BC-1 gave a lower intensity of staining (compared to IST-4 and IST-9) in the matrix of both GM00097 and G3 cells, but was nevertheless clearly visible. However, there was no staining with BC-1 in C1 cultures, indicating that EDIIIB+ FN is not present in the ECM of these cells at this level of detection.

EDIIIA+ and EDIIIB+ FNs are differentially incorporated into the ECM

The destination of EDIIIA+ and EDIIIB+ FNs secreted by parent and hybrid cells in culture was determined by Western blot analyses of protein collected from culture supernatants (SN) and from the ECM and cellular membranes (ECM) remaining attached to the flask after lysis of the cells (Gospodarowicz et al., 1984). The Western blots shown in Fig. 6A are representative of at least 4 independent experiments. Estimates of the proportions of total FN, EDIIIA+ or EDIIIB+ FNs in ECM and the supernatants were made by comparison with the FN band, which comprises FN subunits between approximately 220 and 250 kDa that do not separate sufficiently in these gels to enable resolution of individual FNs (Fig. 6A). This variation in size exists because there are FN isoforms that differ in the IIICS region of the protein in addition to the EDIIIA and EDIIIB variants. Actin (42-43 kDa) was detected with anti-actin mAbs to provide a protein loading control for ECM samples. The anti-actin mAbs also bound to a secreted 60 kDa

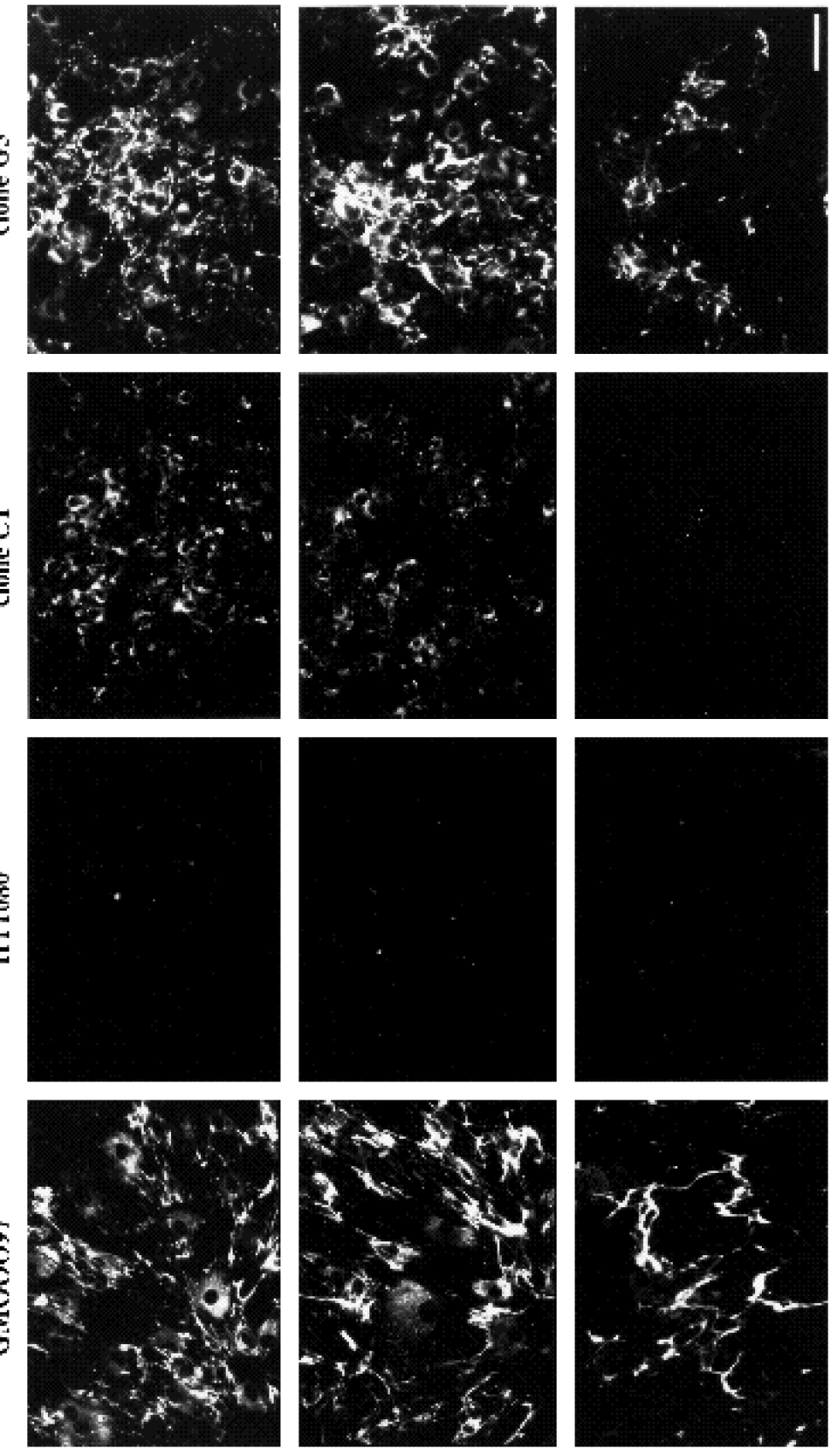

Fig. 5. Localisation of total FNs, EDIIIA+ FN and EDIIIB+ FN by the use of domain-specific mAbs IST-4, IST-9 and B-C1 and indirect immunofluorescence in fixed cells. Bar, 40 μm.

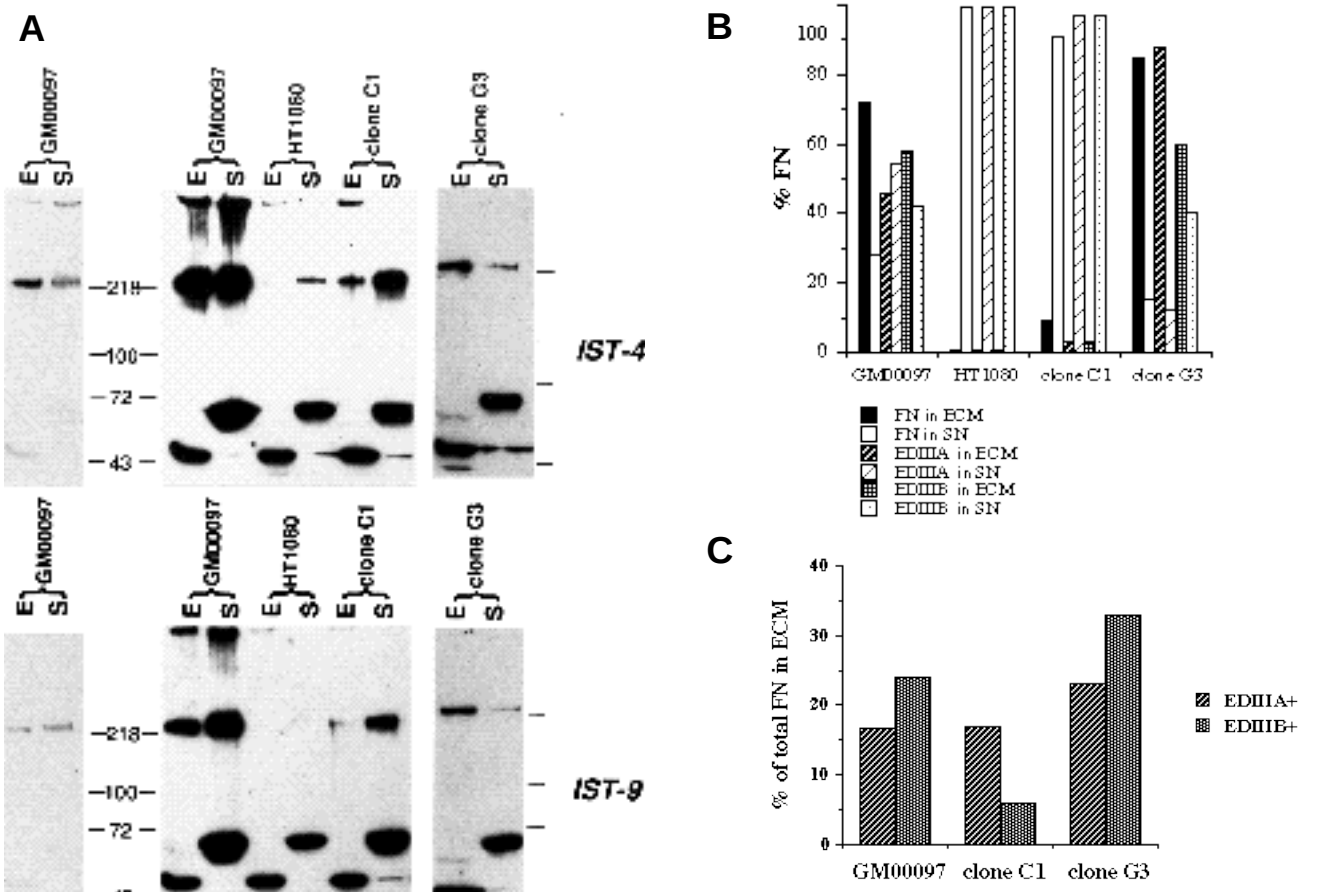

Fig. 6. (A) Western blots of protein extracted from ECM (E), or culture supernatants (S). E tracks were loaded with 5 µl (GM00097 and G3 cells) or 10 µl (HT1080 and C1 cells) protein and S tracks with 5 µl (GM00097 and G3 cells) or 1 µl (HT1080 and C1 cells) protein, out of a total of 100 µl from each 80 cm^2 culture flask. Domain-specific mAbs detected total FN (IST-4), EDIIIA+ FNs (IST-9) or EDIIIB+ FNs (B-C1) and anti-actin mAbs detected actin and the 60 kDa actin-related protein, by ECL. Bands corresponding to FN subunits are 220-250 kDa and actin is 42-43 kDa. The autoradiographs of the Western blots of GM00097 cells on the left of the photograph are shorter exposures of those on the right. Protein standard markers (in kDa) are shown. (B) Densitometric analyses of the autoradiographs shown in A, representative of several independent experiments. ECM proteins are represented by dark-shaded columns and culture supernatants by light-shaded columns. (C) Densitometric analyses of the autoradiographs shown in A, representing the percentage of total FN in the ECM that is EDIIIA+ or EDIIIB+.

protein that has been shown to have epitopes related to actin (Varma et al., 1987). This protein provided a loading control for the culture supernatants. The densitometric analysis of the data shown in Fig. 6A, corrected for loading differences, is graphically represented in Fig. 6B, in which ECMs are represented by dark-shaded columns and supernatants by light-shaded columns and Fig. 6C. All of the FNs released by these cells in culture were either in the ECMs or in the supernatants and no FN was detected by Western blotting of the cell lysate (see Materials and Methods; data not shown).

These data suggest that of the total amount of FN produced by GM00097 cells more than 70% accumulated in the ECM and the remaining 30% or so was released into the supernatant. The short exposure of the GM00097 tracks in Fig. 6A clearly shows that more FN was detected in the ECM than in the supernatant. The pattern is similar in G3 cells in which a large proportion (>80%) of the total FN was detected in the ECM. This pattern of FN distribution was reversed for the C1 cells. Only a small proportion of the total FN (<10%) was detected in the ECM and the rest was released into the supernatant. FN was barely detectable

in the ECM of HT1080 cells. Approximately half (>40%) of EDIIIA+ FN secreted by GM00097 cells accumulated in the ECM, and ECM from G3 cultures contained approximately 90% of the EDIIIA+ FN. In C1 cultures almost all (>90%) of the EDIIIA+ FN was released into the supernatant. EDIIIA+ FN was not detected in the ECM of HT1080 cells. The distribution of EDIIIB+ FN was similar in GM00097 and G3 cells. Both GM00097 and G3 cultures contained more EDIIIB+ FN (approximately 60%) in the ECM than in the supernatant. In C1 cultures almost all of the EDIIIB+ FN was detected in the supernatant; less than 2% was detected in the ECM. EDIIIB+ FN was not detected in the ECM of HT1080 cells. The amount of EDIIIA+ and EDIIIB+ FNs expressed as a percentage of the total FN incorporated into the ECM of GM00097, C1 and G3 cells is represented in Fig. 6C. In both GM00097 and G3 cells, EDIIIB+ formed a higher proportion of the total FN detected in the ECM (24% for GM00097 and 33% for G3 cells) than to EDIIIA+ (16% for GM00097 and 24% for G3 cells). The proportion of EDIIIB+ FN detected in the ECM of C1 cells was at least 4-fold lower (6%) than that in the ECM of GM00097 and G3 cells, whereas the percentage of EDIIIA+ FN was about the same (17%).

DISCUSSION

The function of EDIIIA+ and EDIIIB+ FNs in the adhesion of normal and tumorigenic cells is not known. Here we have explored further the relationship between the expression of EDIIIA+ and EDIIIB+ FNs and tumorigenesis by the use of human somatic cell hybrids made from the fusion of normal fibroblasts and fibrosarcoma-derived cells. Our analyses of GM00097 and HT1080 parent cells, the tumorigenic hybrid clone C1 and the non-tumorigenic hybrid clone G3 revealed that tumorigenicity is not correlated with alternative splicing of FN pre-mRNA or with secretion of specific FN EDIIIA+ or EDIIIB+ protein variants, but it is correlated with the inability of the cell to incorporate specific FN variants into the ECM.

Since the requirement for FN-mediated adhesion is altered in other tumorigenic hybrid cells (Der and Stanbridge, 1980), it was appropriate to examine FN gene expression in the parental and hybrid cells used in our studies. Our data suggest that expression of the FN gene, as indicated by levels of FN mRNA, is not down-regulated in the tumorigenic clone C1 relative to the non-tumorigenic clone G3. Furthermore, there was very little difference between the cells in the ratio of EDIIIA+ to EDIIIA- and EDIIIB+ to EDIIIB- mRNAs generated by alternative splicing (Fig. 4C). Several authors have previously observed differences in alternative splicing of EDIIIA and EDIIIB between normal and transformed or tumorigenic cells. Oncogenic transformation of normal fibroblasts has been reported to induce a slight increase or decrease in either EDIIIA+ or EDIIIB+ FN mRNAs (Schwarzbauer et al., 1987; Magnuson et al., 1991), a significant increase in EDIIIB+ FN mRNA, (Zardi et al., 1987; Borsi et al., 1992), or no change in either (Norton and Hynes, 1987). Similarly, levels of EDIIIA+ and EDIIIB+ FN mRNAs in tumour tissues can be higher or lower than the normal tissue from which the tumours were derived (Oyama et al., 1989, 1990). Differences in splicing of EDIIIA and EDIIIB exons previously observed in tumour-derived cells in culture and the data we report here suggest that alternative FN pre-mRNA splicing is not correlated with tumorigenicity, but may be regulated according to other factors such as the origin of the cell or the transforming agent.

However, the pattern of staining we obtained with immunohistochemical localisation of EDIIIA+ and EDIIIB+ FNs in fixed cells indicates that the presence of the variant FN proteins in the cell layer is correlated with the tumorigenicity of the cell. These data do not appear to agree with previous reports on the distribution of FN variant proteins in transformed and tumour-derived cells. Levels of EDIIIA+ FNs have been reported to be elevated in transformed and tumorigenic cells (Borsi et al., 1987; Vartio et al., 1987) whereas in our study, EDIIIA+ FN is less abundant in the tumorigenic hybrid cells than in the non-tumorigenic hybrid and normal parent cells (Fig. 5).

More striking is the disparity between the immunolocalisation data presented here and the observations of others on the expression of EDIIIB+ FNs in transformed and tumour cells. It has been demonstrated that transformed and tumour-derived cells secrete high levels of EDIIIB+ FNs relative to normal fibroblasts and that EDIIIB+ FNs are present in carcinoma and sarcoma tumour tissue but not in normal adult tissues (Carnemolla et al., 1989; Nicolò et al., 1990; Borsi et al., 1992). These findings have led to the suggestion that EDIIIB+ FN may be a transformation- or tumour-associated form of FN. Our results clearly show that EDIIIB+ FN is substantially reduced in the ECM of the tumorigenic cells in culture. On the contrary, EDIIIB+ FN is abundant in the ECM of both the normal fibroblasts and the non-tumorigenic hybrids.

Closer examination of the destination of FN variants in the parental and hybrid cell cultures, as detected by Western blots of protein extracted from ECM and culture supernatants, provides an explanation for these apparent inconsistencies. We observed a dramatic difference between the tumorigenic and non-tumorigenic cells in the proportions of EDIIIA+ and EDIIIB+ FNs that are incorporated into the ECM compared to what is released into the culture supernatants (Fig. 6A). Most of the FNs produced both by the parental GM00097 fibroblasts and the non-tumorigenic G3 cells are incorporated into the ECM, whereas the converse is true for the HT1080 parent cell and the C1 tumorigenic hybrid. Similar results to those obtained for HT1080 and C1 cells have been reported for transformed cell lines (Hayman et al., 1981; Wagner et al., 1981). A similar proportion of the total FNs in the ECM of GM00097, C1 and G3 cells is EDIIIA+. However, the proportion of EDIIIB+ FN in the ECM of C1 cells as detected by Western blotting is at least 4-fold lower than that in GM00097 and G3 cell ECM (Fig. 6c). In these hybrid cells tumorigenicity is thus accompanied by the inability of the cell to incorporate specific EDIIIB+ FN variants, generated by alternative splicing, into the ECM efficiently. Our results further indicate that the incorporation of EDIIIA+ and EDIIIB+ FNs into the ECM is governed by regulatory mechanisms. Two such mechanisms suggest themselves. One is that there is degradation of FNs by variant-specific proteases produced

by tumorigenic cells. Although transformed cells express proteases that degrade FNs (Chen et al., 1984; Chen and Chen, 1987), there is so far no evidence that FN variant-specific proteases are secreted by cells. Furthermore, it is unlikely that intact EDIIIA+ and EDIIIB+ FNs would be detected at high levels in the culture supernatants of tumorigenic cells if they were being specifically degraded. A second possibility is that there is tumour-specific regulation of the expression of cell surface receptors that preferentially bind FN variant proteins. Obvious candidates for such receptors are the integrins (reviewed by Humphries, 1990; Hynes, 1992). One FN receptor, the integrin complex $\alpha_5\beta_1$, has been shown to have some function in the suppression of transformation and tumorigenesis in oncogenically transformed Chinese hamster ovary cells (Giancotti and Ruoslahti, 1990; Schreiner et al., 1991).

We have shown here that the regulation of expression of EDIIIA+ and EDIIIB+ FNs in normal and tumorigenic cells does not necessarily occur at the level of splicing. We further conclude that EDIIIB+ FNs are specifically lost from the surface of tumorigenic somatic cell hybrids and that this loss is due to the failure of EDIIIB+ FN to be incorporated into the ECM of the tumorigenic cells.

We thank Dr L. Zardi for the generous gift of the antibodies, J. Sharps for expert technical assistance, and Dr E. P. Evans and M. Burtenshaw for the cytogenetic analyses. This work was supported by the Cancer Research Campaign.

REFERENCES

Barone, M. V., Henchcliffe, C., Baralle, F. E. and G. Paolella. (1989). Cell type specific trans-acting factors are involved in alternative splicing of human fibronectin pre-mRNA. *EMBO J.* **8**, 1079-1085.

Borsi, L., Balza, E., Allemanni, G. and Zardi, L. (1992). Differential expression of the fibronectin isoform containing the ED-B oncofoetal domain in normal human fibroblast cell lines originating from different tissues. *Exp. Cell Res.* **199**, 98-105.

Borsi, L., Carnemolla, B., Castellani, P., Rosellini, P., Vecchio, D., Allemanni, G., Chang, S. E., Taylor-Papadimitriou, J., Pande, H. and Zardi, L. (1987). Monoclonal antibodies in the analysis of fibronectin isoforms generated by alternative splicing of mRNA precursors in normal and transformed human cells. *J. Cell Biol.* **104**, 595-600.

Borsi, L., Castellani, P., Risso, A. M., Leprini, A. and Zardi, L. (1990). Transforming growth factor-β regulates the splicing pattern of fibronectin messenger RNA precursor. *FEBSMeet*. **261**, 175-178.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G. and Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* **4**, 487-525.

Carley, W. W., Barak, L. S. and Webb, W. W. (1981). F-actin aggregates in transformed cells. *J. Cell Biol.* **90**, 797-802.

Carnemolla, B., Balza, E., Siri, A., Zardi, L. and Nicotra, M. R. (1989). A tumour-associated fibronectin isoform generated by alternative splicing of messenger RNA precursors. *J. Cell Biol.* **108**, 1139-1148.

Chen, J. and Chen, W. (1987). Fibronectin-degrading proteases from the membranes of transformed cells. *Cell* **48**, 193-203.

Chen, L. B., Gallimore, P. H. and McDougall, J. K. (1976). Correlation between tumour induction and the large external transformation sensitive protein on the cell surface. *Proc. Nat. Acad. Sci. USA* **73**, 3570-3574.

Chen, W., Older, K., Bernard, B. A. and Chu, F. (1984). Expression of transformation-associated protease(s) that degrade fibronectin at cell contact sites. *J. Cell Biol.* **98**, 1546-1553.

Chomczynski, P. and Sacchi, N. (1987). Single step method of DNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Der, C. J. and Stanbridge, E. J. (1978). Lack of correlation between the decreased expression of cell surface LETS protein and tumorigenicity in human cell hybrids. *Cell* **15,** 1241-1251.

Der, C. J., Ash, J. F. and Stanbridge, E. J. (1981). Cytoskeletal and transmembrane interactions in the expression of tumorigenicity in human cell hybrids. *J. Cell Sci.* **52**, 151-166.

DeSimone, D. W., Norton, P. A. and Hynes, R. O. (1992). Identification and characterization of alternatively spliced fibronectin mRNAs expressed in early *Xenopus* embryos. *Dev. Biol.* **149**, 357-369.

Evans, E. P., Burtenshaw, M. D., Brown, B. B., Hennion, R. and Harris, H. (1982). The analysis of malignancy by cell fusion. IX. Re-examination and clarification of the cytogenetic problem. *J. Cell Sci.* **56**, 113-130.

Gallimore, P. H., McDougall, J. K. and Chen, L. B. (1977). In vitro traits of adenovirus-transformed cell lines and their relevance to tumorigenicity. *Cell* **10**, 669-678.

Giancotti, F. G. and Ruoslahti, E. (1990). Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of chinese hamster ovary cells. *Cell* **60**, 849-859.

Gospodarowicz, D., Lepine, J., Massoglia, S. and Wood, I. (1984). Comparison of the ability of basement membranes produced by corneal endothelial and mouse-derived endodermal PF-HR-9 cells to support the proliferation and differentiation of bovine kidney tubule epithelial cells in vitro. *J. Cell Biol.* **99**, 947-961.

Harris, H. (1988). The analysis of malignancy by cell fusion: the position in 1988. *Cancer Res.* **48**, 3302-3306.

Harris, H. (1990). Tumor suppressor genes: studies with mouse hybrid cells. In *Tumor Suppressor Genes* (ed. G. Klein). Marcel Dekker, Inc., New York and Basel.

Harris, H. and Watkins, J. F. (1965). Hybrid cells derived from mouse and man; artificial heterokaryons of mammalian cells from different species. *Nature* **205**, 640-646.

Hayman, E. G., Engvall, E. and Ruoslahti, E. (1981). Concomitant loss of cell surface fibronectin and laminin from transformed rat kidney cells. *J. Cell Biol.* **88**, 352-357.

Humphries, M. J. (1990). The molecular basis and specificity of integrin-ligand interactions. *J. Cell Sci.* **97**, 585-592.

Humphries, M. J., Komoriya, A. Akiyama, S. K,. Olden, K. and Yamada, K. M. (1987). Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion. *J. Biol. Chem.* **262**, 6886-6892.

Hynes, R. O. (1973). Alteration of cell surface proteins by viral transformation and by proteolysis. *Proc. Nat. Acad. Sci. USA* **70**, 3170-3174.

Hynes, R. O. (1985). Molecular biology of fibronectin. *Annu. Rev. Cell Biol*. **1**, 67-90.

Hynes, R. O. (1990). *Fibronectins*, Springer Verlag, Berlin.

Hynes, R. O. (1992). Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**, 11-25.

Kornblihtt, A. R., Vibe-Pedersen, K. and Baralle, F. E. (1984a). Human fibronectin: cell specific alternative mRNA splicing generates polypeptide chains differing in the number of internal repeats. *Nucl. Acids Res*. **12**, 5853-5868.

Kornblihtt, A. R., Vibe-Pedersen, K. and Baralle, F. E. (1984b). Human fibronectin: molecular cloning evidence for two mRNA species differing by an internal segment coding for a structural domain. *EMBO J.* **3**, 221-226.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **27**, 680-685.

Littlefield, J. W. (1964). Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. *Science* **145**, 709-710.

Magnuson, V. L., Young, M., Schattenberg, D. G., Mancini, M. A., Chen, D., Steffensen, B. and Klebe, R. J. (1991). The alternative splicing of fibronectin pre-mRNA is altered during aging and in response to growth factors. *J. Biol. Chem.* **266**, 14654-14662.

Mardon, H. J., Bee, J., von der Mark, K. and Owen, M. E. (1987). Development of osteogenic tissue in diffusion chambers from early precursor cells in bone marrow of adult rats. *Cell Tiss. Res.* **25**, 157-165.

Mardon, H. J. and Sebastio, G. (1992). Regulation of alternative splicing in the IIICS region of human fibronectin pre-mRNA encoding cell binding sites CS1 and CS5. *J. Cell Sci.* **103**, 423-433.

Nicolò, G., Salvi, S., Oliveri, G., Borsi, L., Castellani, P. and Zardi, L. (1990). Expression of tenascin and of the ED-B containing oncofetal fibronectin isoform in human cancer. *Cell Differ. Dev.* **32**, 401-408.

Norton, P. A. and Hynes, R. O. (1987). Alternative splicing of chicken

fibronectin in embryos and in normal and transformed cells. *Mol. Cell. Biol*. **7**, 4297-4307.

Owens, R. J., Kornblihtt, A. R. and Baralle, F. E. (1986). Fibronectin, the generation of multiple polypeptides from a single gene. In *Oxford Surveys on Eukaryotic Genes* (ed. N. Maclean), vol. 3, pp, 141-160.

Oyama, F., Hirohashi, S., Shimosato, Y., Titani, K. and Sekiguchi, K. (1989). Deregulation of alternative splicing of fibronectin pre-mRNA in malignant human liver cells. *J. Biol. Chem.* **264**, 10331-10334.

Oyama, F., Hirohashi, S., Shimosato, Y., Titani, K. and Sekiguchi, K. (1990). Oncodevelopmental regulation of the alternative splicing of fibronectin pre-messenger RNA in human lung tissues. *Cancer Res*. **50**, 1075-1078.

Pollack, R., Osborn, M. and Weber, K. (1975). Patterns of organisation of actin and myosin in normal and transformed cultured cells. *Proc. Nat. Acad. Sci. USA* **994**, 994-998.

Proudfoot, N. J., Rutherford, T. R. and Partington, G. A. (1984). Transcriptional analysis of human ζ-globin genes. *EMBO J*. **3**, 1533-1540.

Punnett, H. H., Kistermacherm M. L., Greene, A. E. and Coriell, L. L. (1974). An (X;1) translocation, balanced 46 chromosomes. Repository identification no. GM-97. *Cytogenet. Cell Genet.* **13**, 406-407.

Rahilly, M. A., Salter, D. M. and Fleming, S. (1991). Composition and organisation of cell-substratum contacts in normal and neoplastic renal epithelium. *J. Pathol*. **165**, 163-171.

Rasheed, S., Nelson-Rees, W. A., Toth, E. M., Arnstein, P. and Gardner, M. B. (1974). Characterisation of a newly derived human sarcoma cell line (HT-1080). *Cancer* **33**, 1027-1033.

Schreiner, M. F., Fisher, M., Hussein, S. and Juliano, R. L. (1991). Increased tumorigenicity of fibronectin receptor deficient chinese hamster ovary cell variants. *Cancer Res.* **51**, 1738-1740.

Schwarzbauer, J. E., Patel, R. S., Fonda, D. and Hynes, R. O. (1987). Multiple sites of alternative splicing of the rat fibronectin gene transcript. *EMBO J*. **6**, 2573-2580.

Schwarzbauer, J. E., Tamkun, J. W., Lemischka, I. R. and Hynes, R. O. (1983). Three different fibronectin mRNAs arise by alternative splicing within the coding region. *Cell* **35**, 421-431.

Sekiguchi, K., Siri, A., Zardi, L. and Hakomori, S. (1985). Differences in domain structure between human fibronectins isolated from plasma and from culture supernatants of normal and transformed fibroblasts. *J. Biol. Chem*. **260**, 5105-5114.

Steel, D. M. and Harris, H. (1989). The effect of antisense RNA to fibronectin on the malignancy of hybrids between melanoma cells and normal fibroblasts. *J. Cell Sci.* **93**, 515-524.

Tamkun, J. W., Schwarzbauer, J. E. and Hynes, R. O. (1984). A single rat fibronectin gene generates three different mRNAs by alternative splicing of a complete exon. *Proc. Nat. Acad. Sci. USA* **81**, 5140-5144.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.

Varma, M., Aebi, U., Fleming, J. and Leavitt, J. (1987). A 60 kDa polypeptide in mammalian cells related to epitopes. *Exp. Cell Res*. 173, 163-173.

Vartio, T., Laitinen, L., Narvanen, O., Cutolo, M., Thornel, L., Zardi, L. and Virtanen, I. (1987). Differential expression of the ED sequence-containing form of cellular fibronectin in embyronic and adult human tissues. *J. Cell Sci.* **88**, 419-430.

Vibe-Pedersen, K., Kornblihtt, A. R. and Baralle, F. E. (1984). Expression of a human α-globin/fibronectin gene hybrid generates two mRNAs by alternative splicing. *EMBO J*. **3**, 2511-2516.

Wagner, D. D., Ivatt, R., Destree, A. T. and Hynes, R. O. (1981). Similarities and differences between the fibronectins of normal and transformed hamster cells. *J. Biol. Chem.* **256**, 11708-11715.

Yamada, K. M., Yamada, S. S. and Pastan, I. (1976). Cell surface protein partially restores morphology, adhesiveness and contact inhibition of movement to transformed fibroblasts. *Proc. Nat. Acad. Sci. USA* **73**, 1217-1221.

Zajchowski, D. A., Band, V., Trask, D. K., Kling, D., Connolly. J. L. and Sager, R. (1990). Suppression of tumor-forming ability and related traits in MCF-7 human breast cancer cells with immortal mammary epithelial cells. *Proc. Nat. Acad. Sci. USA* **87**, 2314-2318.

Zardi, L., Carnemolla, B., Siri, A., Petersen, T. E., Paolella, G., Sebastio, G. and F. E. Baralle. (1987). Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. *EMBO J*. **6**, 2337-2342

(Received 6 October 1992 - Accepted 24 November 1992)