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Reappearance of an Embryonic Pattern of Fibronectin Splicing during Wound Healing in the Adult Rat

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Abstract. The adhesive extracellular matrix glycoprotein fibronectin (FN) is thought to play an important role in the cell migration associated with wound healing. Immunolocalization studies show abundant FN in healing wounds; however, these studies cannot define the cellular site(s) of FN synthesis, nor do they distinguish the different and potentially functionally distinct forms of FN that can arise from alternative splicing of the primary gene transcript. To examine these questions of FN synthesis and splicing during wound healing, we have performed in situ hybridization with segment-specific probes on healing wounds in adult rat skin. We find that the FN gene is expressed at increased levels after wounding both in the cells at the base of the wound and in subjacent muscle and dermis lateral to the wound. Interestingly, however, the pattern of splicing of FN mRNA was different in these areas. In adjacent dermis and muscle, the splicing pattern remains identical with that seen in normal adult rat skin, with two of the three spliced segments (EIIIA and EIIIB) excluded from FN mRNA. In contrast, these two segments are included in the FN mRNA present in the cells at the base of the wound. As a result, the mRNA in this region is spliced in a pattern identical with that found during early embryogenesis. The finding that the pattern of FN splicing during wound healing resembles an embryonic pattern suggests that alternative splicing may be used during wound healing as a mechanism to generate forms of FN that may be functionally more appropriate for the cell migration and proliferation associated with tissue repair.

THE ability to repair damaged tissue, wound healing, represents an important response to injury that is common to all complex organisms. Just as in embryonic development, this process involves cell proliferation, migration, and differentiation of a number of different cell types (Clark and Henson, 1988). It therefore seems likely that wound healing might share many of the molecular mechanisms used during development. A number of studies have examined the role of the adhesive extracellular matrix glycoprotein fibronectin (FN),[1] which has been shown to play an important role in embryonic cell migration. FN promotes cell migration in culture (Ali and Hynes, 1978; Rovasio et al., 1983) and is present in the embryo associated with many different cell migrations (Critchley et al., 1979; Kurkinen et al., 1979; Newgreen and Thiery, 1980; Mayer et al., 1981; Heasman et al., 1981; Duband and Thiery, 1982; Thiery et al., 1982; Linask and Lash, 1986; Kitten et al., 1987; Dufour et al., 1988). In addition, antibodies to FN (Boucaut et al., 1984; Poole and Thiery, 1986) or to cell surface receptors of the integrin glycoprotein family (Bronner-Fraser,

1985, 1986) can block migration when injected into the intact embryo.

FN is expressed at high levels in healing wounds (Kurkinen et al., 1980; Grinnell et al., 1981; Clark et al., 1982a, b) and is derived from two sources: plasma FN (pFN), which is present in the exudate from damaged blood vessels; and "cellular" FN, which is synthesized locally in the wound tissue (Clark et al., 1983). It seems likely that FN plays an important role in wound healing as it appears to be involved in the migration in vitro of four major cell types that migrate into the area of the wound. Fibroblasts and epidermal cells (keratinocytes) are stimulated to migrate by FN (Ali and Hynes, 1978; Donaldson and Mahan, 1983; Takashima et al., 1986). FN is a chemoattractant stimulus for endothelial cells (Bowersox and Sorgente, 1982), whereas FN fragments are chemotactic for macrophages (Norris et al., 1982; Clark et al., 1988).

The antibody localization methods used to demonstrate FN in healing tissue do not allow a more precise definition of the role of FN. First, identification in wounds of an extracellular molecule does not distinguish between local secretion and extravasation from plasma. Second, it is likely that multiple forms of FN are present, as the primary FN gene transcript can be alternatively spliced in three regions. These multiple forms may each play different roles in cell movement, but can not be distinguished by the antibodies

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1. *Abbreviations used in this paper*: EIIIA and EIIIB, spliced segments of type III repeats; FN, fibronectin; nt, nucleotide; V, nonhomologous segment lying between two type III repeats.

presently used to identify FN in wounds, which were prepared against intact FN and will therefore recognize epitopes present on all forms of FN. The potential molecular heterogeneity resulting from alternative splicing of mRNA for rat FN is illustrated in Fig. 1. The three spliced segments are shown; two, designated EIIIA and EIIIB, represent part of the series of so-called type III repeats that constitute the central portion of the molecule while the third, V, represents a nonhomologous segment lying between two type III repeats (Hynes, 1985). EIIIA and EIIIB can either be included or excluded, whereas the V region in the rat can be partially or completely included (depending on the choice of splice site used) to generate inserts of 95 or 120 amino acids (Fig. 1) (Schwarzbauer et al., 1983; Hynes, 1985; Kornblihtt et al., 1985; Paul et al., 1986; Schwarzbauer et al., 1987; Norton and Hynes, 1987). As a result of this splicing, 12 potential forms of rat FN can be generated. At least in the human and chicken, the potentially excluded V region contains cell adhesion sites (Humphries et al., 1986, 1987, 1988), suggesting that some of these different forms may be functionally distinct from each other.

In this study, we have addressed these questions of synthesis and splicing during wound healing by using in situ hybridization with probes that either recognize all forms of FN mRNA or that are specific for the different spliced variants. We find that the level of FN mRNA present in wounds is much greater than that found in normal adult rat skin. Also, the FN mRNA in the wound shows a pattern of splicing different from that in normal skin and similar to that found in the early embryo. As well as allowing a more precise definition of the patterns of FN synthesis in wound repair, these results suggest that FN splicing is used as a mechanism to create functionally appropriate forms of FN that facilitate the process of wound healing.

Materials and Methods

Preparation of Tissue

4-mm punch biopsy lesions were made in the flanks of female adult rats (150–200 g, CD rats, Charles River Laboratories, Inc., Wilmington, MA), which had been anaesthetised with 25 mg/kg ketamine HCl and 5 mg/kg xylazine. At intervals (1, 2, 4, 7 and 14 d) after biopsy, rats were killed and wound sites were excised. Individual rats were used for each wound and control tissue was taken from unwounded rats.

Excised wound tissue was immersed in ice-cold 5% glacial acetic acid, 4% formaldehyde, and 85% ethanol for 15–30 min and bisected; fixation was continued for a total of 3–4 h. Tissues were then dehydrated in ascending concentrations of ethanol and embedded in paraffin (Paraplast Xtra; Polysciences, Inc., Warrington, PA). 7-μm microtome sections were mounted on glass slides that had been pretreated with 10% TESPA (3-aminopropyl-triethoxysilane) in toluene for 1 h at 80°C, washed in toluene, and activated by immersion in 4% formaldehyde for 1 h (after Berger, 1986, modified as suggested by Dr. D. Melton, Harvard University, Cambridge, MA). The slides were left on a warm plate at 45°C overnight before use.

Preparation of Probes for In Situ Hybridization

To synthesize the probes used in this study, fragments of DNA from larger clones and corresponding either to a region in the type-I repeats near the COOH-terminal end of FN always included in the final transcript, or to parts of the EIIIA-, EIIIB-, or V95-spliced regions were subcloned into pGEM plasmids to allow the synthesis of both sense and antisense probes. The lengths of the DNA inserts were 270, 213, 209, and 250 nucleotides (nt) for the segments C, EIIIA, EIIIB, and V95, respectively (see Fig. 1).

Single-stranded RNA probes labeled to a specific activity of ∼10⁸ cpm/μg with [³⁵S]-UTP were synthesized using a commercially available transcription kit (Promega Biotec, Madison, WI). After the synthesis, all probes were purified on denaturing polyacrylamide gels and used without any reduction in length by alkaline hydrolysis (Angerer et al., 1987).

In Situ Hybridization

Prehybridization and hybridization were performed as previously described (ffrench-Constant and Hynes, 1988). In brief, slides were dewaxed and sequentially passed through 0.2 M HCl, 1 μg/ml proteinase K, 0.2% glycine, 4% paraformaldehyde, and 1/200 (vol/vol) acetic anhydride in 0.1 M triethanolamine, pH 8.0, before addition of the hybridization mixture. Hybridization buffer (50% deionized formamide, 10% dextran sulfate, 0.3 M NaCl, 10 mM Tris [pH 7.6], 5 mM EDTA, 0.02% [wt/vol] Ficoll 400, 0.02% [wt/vol] polyvinylpyrolidone, 0.02% [wt/vol] BSA, 10 mM DTT, 100 μg/ml yeast tRNA, and 500 μM nonradiolabeled thio-UTP) containing the appropriate probe at a concentration of 0.3 μg/ml per kb probe complexity was adjusted to a pH of 6.0 and placed on the sections. This probe concentration is the same as that used in our studies on FN mRNA distribution in chicken embryos (ffrench-Constant and Hynes, 1988, 1989). In those studies we found that higher probe concentrations produced higher backgrounds without any further increase in signal (unpublished observations). 0.3 μg/ml per kb probe complexity therefore represents a suitable probe concentration that is sufficient to saturate target FN mRNAs even in early embryonic cell types that express abundant FN mRNA (ffrench-Constant and Hynes, 1988), and for this reason it was chosen for the present study on wound healing. After hybridization overnight at 50°C, slides were rinsed in 50% formamide, 2× SSC, 10 mM DTT at 50°C, digested with 10 μg/ml RNase A (type IIIA; Sigma Chemical Co., St. Louis, MO), and then washed in 50% formamide, 2× SSC, 10 mM DTT at 65°C as before. Slides were then dried, dipped in Kodak NTB-2 emulsion (diluted 1:1 in water) and exposed for 7 d at −20°C, after which time they were developed in Kodak D19, fixed, stained with 0.02% toluidine blue, and mounted with DPX mountant. Sections were viewed on Zeiss Universal or Axiophot microscopes equipped with brightfield and darkfield optics, and photographed on Tech Pan film at 85ASA.

Results

FN Gene Expression and Splicing in Normal Skin

To determine the localization of FN mRNA in rat tissue sections, we hybridized these sections with an antisense ³⁵S-labeled RNA probe synthesized as described in Materials and Methods. The template used was a 270-nt segment corresponding to a region in the type-I repeats near the COOH-terminal end of the molecule (Fig. 1). This region is included in all known forms of FN mRNA and the antisense probe can therefore be used to detect all the different spliced variants of FN mRNA.

When this probe (designated FN-C) was hybridized to sections of normal skin, specific labeling of scattered cells was observed in the deep (reticular) dermis and in the subjacent adipose tissue (Fig. 2). Often, labeled cells were found surrounding the base of hair follicles (not shown). In addition, labeled cells were seen in a narrow band just superficial to the striated muscle layer (panniculus carnosus) and occasionally between fibers within this muscle itself (Fig. 2). More extensive labeling was present in the thin layer of connective tissue deep to the muscle layers (Fig. 2). The epidermis and superficial (papillary) dermis were not labeled. The specificity of this hybridization pattern was confirmed by the absence of labeling with a sense probe synthesized from the same construct (data not shown).

We next determined the splicing pattern of FN mRNA in normal skin. Sections were incubated with antisense probes synthesized using DNA fragments from the EIIIA, EIIIB, and V95 regions as templates (probes designated FN-EIIIA, FN-EIIIB, and FN-V95, as shown in Fig. 1). These probes
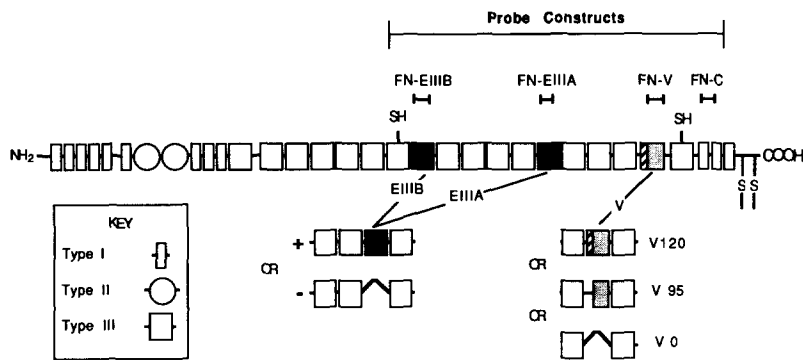
Figure 1. A diagrammatic representation of rat FN showing the three regions of alternative splicing and illustrating the potential heterogeneity that can be generated by this mechanism. The four probes used in this study are shown; note that the V95 probe cannot distinguish the V95 and V120 forms of FN.

were of similar length (213, 209, and 250 nt, respectively) to the 270-nt FN-C probe. As shown in Fig. 1, the V95 region is included in the mRNA encoding both the 95- and 120-amino acid insert, so these two forms cannot be distinguished by the FN-V95 probe.

No significant labeling was found with the FN-EIIIA or FN-EIIIB probes, whereas the FN-V95 probe labeled normal skin in a pattern similar to that seen with the FN-C probe (Fig. 2). No labeling of sections of normal skin was seen with a sense probe synthesized from the FN-V95 construct (not

shown). All these probes are of similar length and specific activity and were used at the same concentration in the hybridization reactions. The absence of labeling with FN-EIIIA and FN-EIIIB indicates, therefore, that the majority of the FN mRNA that is present in scattered cells within the dermis and muscle of normal adult rat skin lacks these two segments. In contrast, the V segment is included in this FN mRNA. However, the semiquantitative nature of in situ hybridization does not allow an accurate determination of the extent of this inclusion.
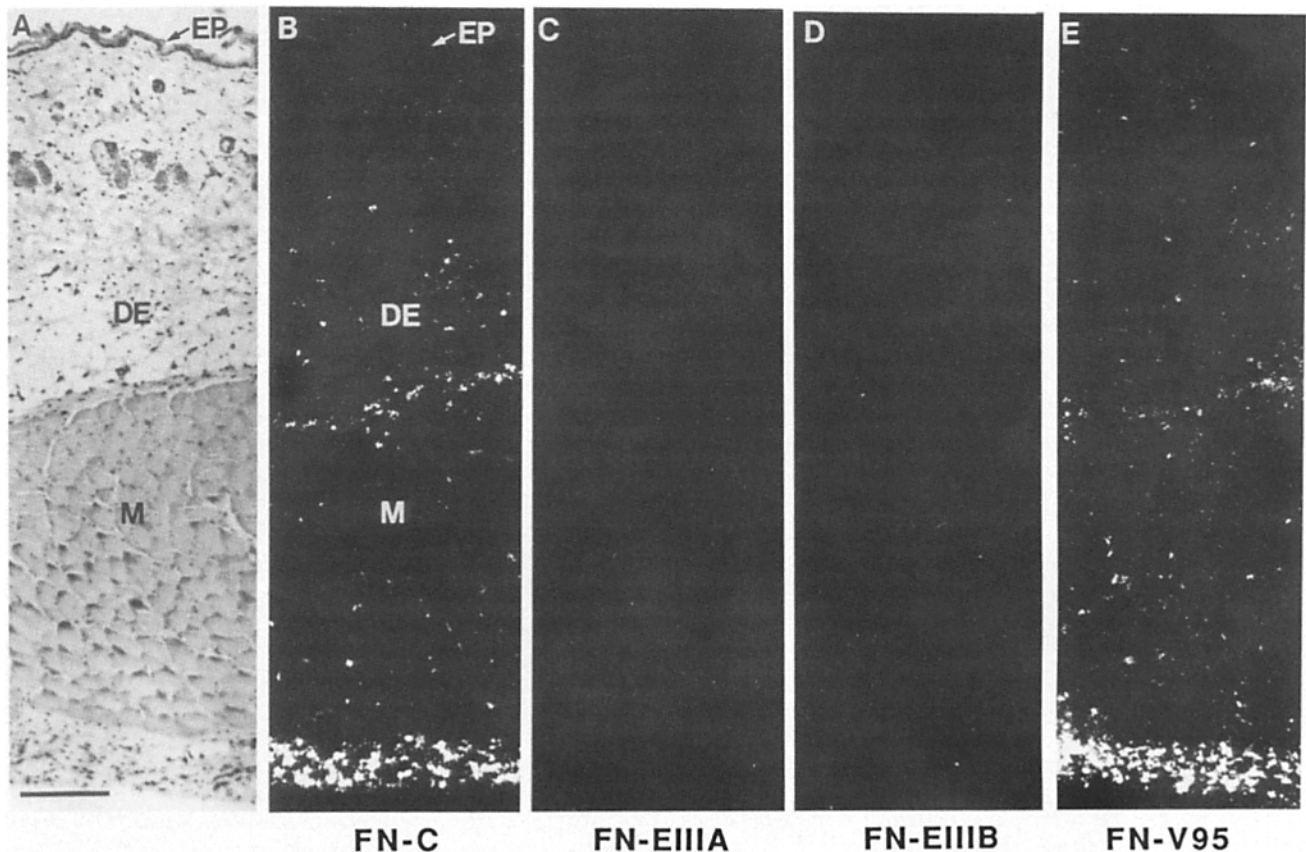

Figure 2. Four closely adjacent sections of normal adult rat skin hybridized with the FN-C, FN-EIIIA, FN-EIIIB, and FN-V probes as indicated, and then viewed with darkfield optics (*B–E*). A representative section viewed with brightfield optics is shown (*A*). Scattered labeling with FN-C is seen in the reticular dermis (*DE*), whereas the epidermis (*EP*) is unlabeled. Labeling is also seen on either side of the striated muscle layer (*M*) below the dermis, and occasionally within the muscle itself. The segment-specific probes show an identical pattern of labeling with FN-V, but no labeling with FN-EIIIA or FN-EIIIB, suggesting that the FN mRNA in normal rat skin is $A^-B^-V^+$. Bar, 200 μm.
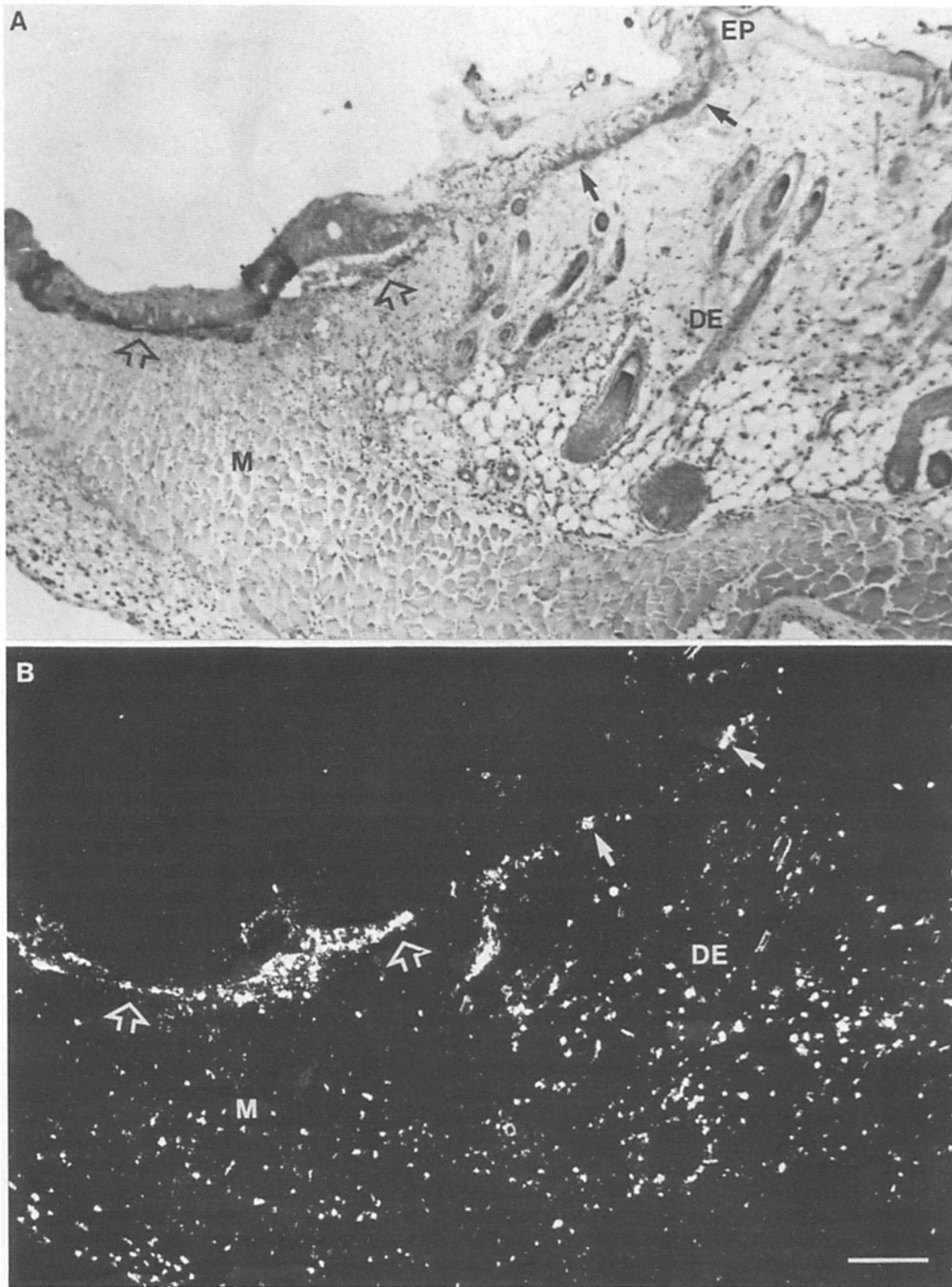
Figure 3. A section through one edge of a 2-d wound, hybridized with FN-C and viewed with brightfield (*A*) and darkfield (*B*) optics. Extensive scattered labeling is seen in the dermis (*DE*) and muscle (*M*). Labeling is also seen at the base (*hollow arrows*) and edge (*arrows*) of the wound as well as in the uninjured dermis lateral to the wound (*DE*). Note that the epidermal cells (*EP*) have not yet started to migrate centrally. They will migrate down the edge and over the base of the wound during healing. Bar, 200 μm.

FN Gene Expression after Wounding

Skin punch biopsy wounds provide a well-defined and reproducible experimental model (Clark and Henson, 1988). Wounding to the level of the deep reticular dermis is followed immediately by the coagulation of blood and extravasated plasma fibrinogen and FN, forming a gel that serves as provisional matrix. This matrix is invaded initially (within hours) by neutrophils and later by monocytes/macrophages; among
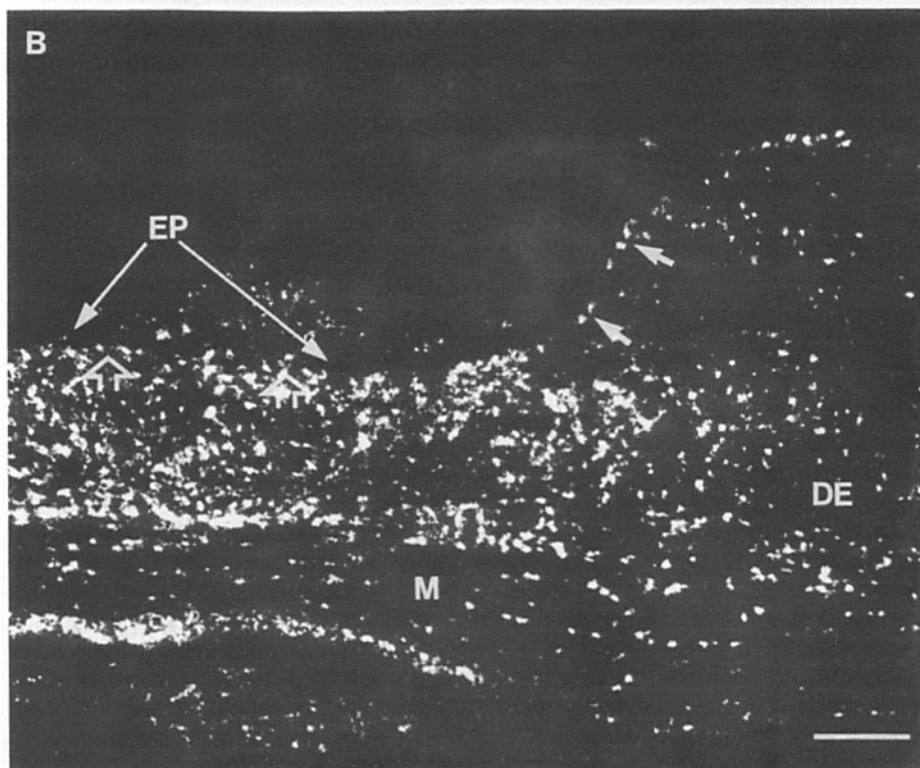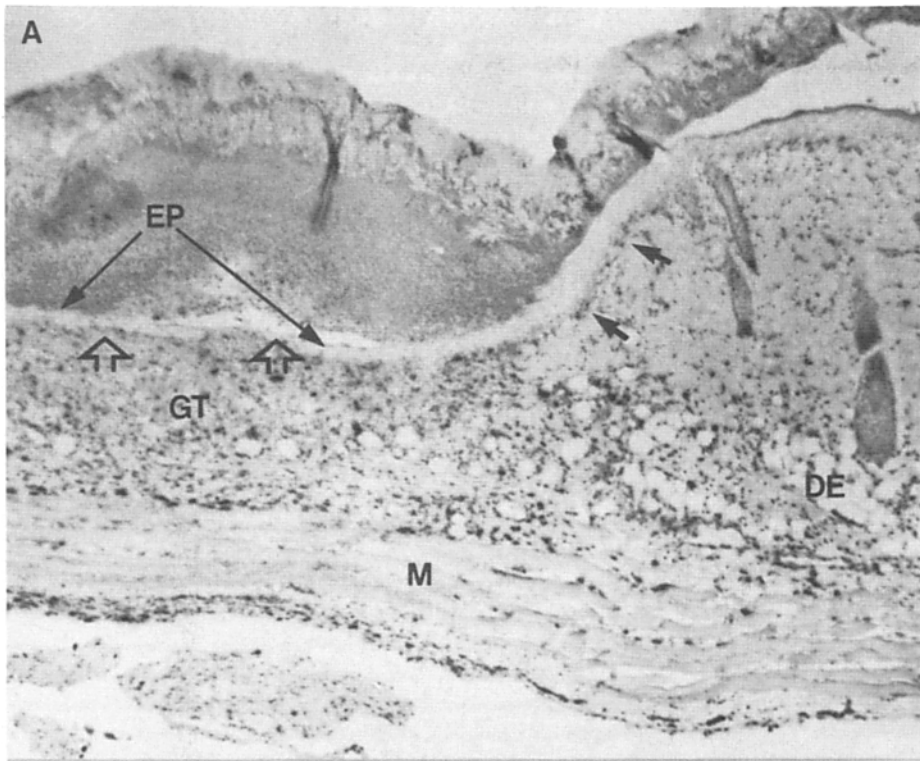
Figure 4. A section through one edge of a 4-d wound hybridized with FN-C and viewed as in Fig. 3. Epidermal cells (*EP*) have now migrated into the base of the wound. As in 2-d wounds, labeled cells are found in the lateral dermis (*DE*), muscle (*M*), base (*hollow arrows*), and edges (*arrows*) of the wound. Note, however, that the labeling of the granulation tissue (*GT*) at the base is more intense and widespread than was seen at 2 d. Bar, 200 μm.

their many functions, these inflammatory cells debride dead tissue and phagocytose bacteria. Epidermal keratinocytes at the wound edge proliferate and migrate inwardly (centripetally) over the provisional matrix, participate in the generation of a new basement membrane, and restore a complete epidermis within 7 d. Inflammatory cells decline in number (over days) as fibroblasts and endothelial cells invade the wound bed, forming highly vascular granulation tissue. Granulation tissue, in turn, is remodeled and, with the laying down of interstitial collagens, is transformed into dense scar tissue (over weeks).

Healing wound sites were examined 1, 2, 4, 7, and 14 d

after wounding, and the distribution of FN mRNA was determined by hybridization with the FN-C probe. At 1 and 2 d (day 2, shown in Fig. 3) the wound, which extended deep into the reticular dermis, was covered with a desiccated blood clot. FN-C labeling was seen in cells that formed a band in the wound bed immediately beneath this clot. In addition, many more labeled cells were seen in the neighboring dermis and subjacent striated muscle than had been observed in normal skin (compare Fig. 3 with Fig. 2). Two differences were observed between the labeling of the wounds at 1 and 2 d. First, labeling was more intense and extensive on day 2. Second, at 2 d, labeling was particularly marked in cells immediately beneath the wound in the region over which the epidermal cells will migrate centripetally to cover the wound (Fig. 3).

At 4 d (Fig. 4) the epidermis had migrated centrally to begin to cover the wound. Widespread and intense cell labeling was seen in the granulation tissue which had now formed at the base of the wound. As at 2 d, numerous labeled cells were seen in the neighboring dermis and muscle (Fig. 4), extending a greater distance from the wound than at 2 d. In addition, labeled cells continued to be present in the superficial dermis at the edge of the wound (Fig. 5). In contrast, however, the epidermal cells themselves were unlabeled at all times (Figs. 4 and 5).

By 7 d (Fig. 6) the wound area was smaller, reflecting some degree of wound contraction, and, in addition, the wound was now closed, being completely covered by epidermal cells. The underlying granulation tissue appeared as a discrete triangular zone rich in blood vessels and newly deposited collagen. Cells of the granulation tissue were still intensely labeled, but labeling was now less extensive in neighboring dermis and muscle than at earlier intervals (compare Fig. 6 with Figs. 3 and 4).

At the latest time studied, 14 d (not shown), labeling of granulation tissue (now still further reduced in area) was considerably less intense than at 7 d and the extent of labeling in adjacent tissues was reduced to normal levels. No labeling of any of these regions at any time during healing was seen with a sense probe synthesized from the FN-C template, confirming the specificity of the labeling.

These results with the FN-C probe therefore demonstrate increased FN mRNA levels after wounding in two locations; in the base and edge of the wound, and in cells present in the uninjured dermis and muscle 1–2 mm lateral to the wound.

FN Splicing after Wounding

The pattern of alternative splicing of FN mRNA in these wounds was determined by hybridizing sections with the FN-EIIIA, FN-EIIIB, and FN-V95 probes (Fig. 7). As in normal skin, FN-V95 labeled wound sections in a pattern identical with that seen with FN-C at all of the time intervals examined (compare Fig. 7 with Fig. 4). In contrast with our results in normal skin, however, labeling was now also found with the FN-EIIIA and FN-EIIIB probes at all the different stages examined (shown for the 4-d wound in Fig. 7). This labeling was largely restricted to the granulation tissue at the base of the wound and to the cells of the superficial dermis at the edge of the wound over which epidermal cell migration occurs. Unlike the results with FN-C or FN-V95, a significantly lower level of labeling was seen in the muscle and in the dermis lateral to the wound (compare Figs. 7 and 8 with Fig. 4). In addition to this restricted distribution of FN-EIIIA and FN-EIIIB labeling, we found that the labeling with FN-EIIIA was always more intense than that with FN-EIIIB (Fig. 7). As these two probes were virtually identical in length,
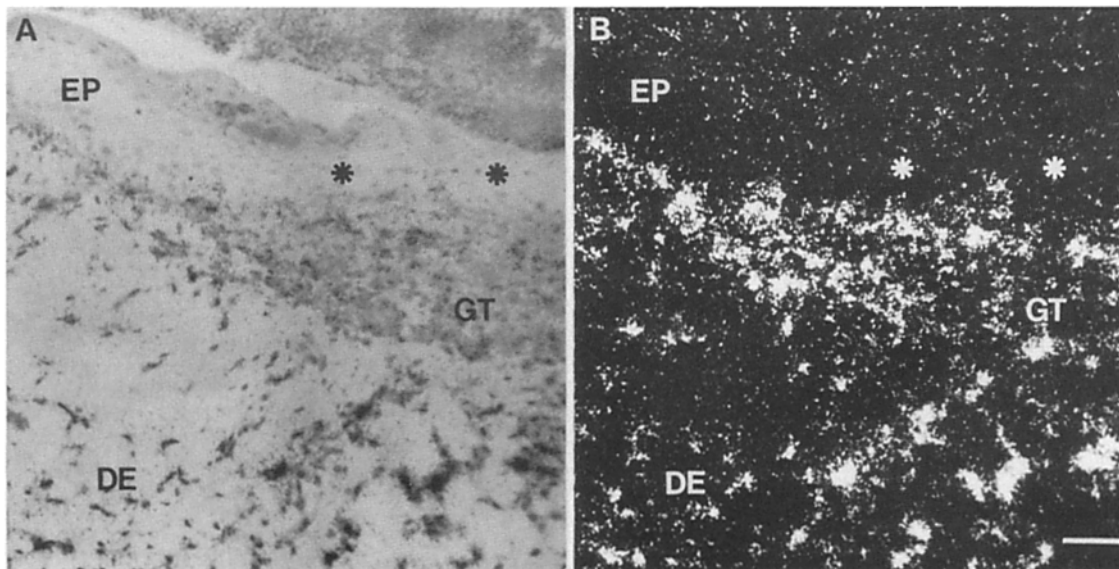

Figure 5. A section of a 4-d wound shown at a higher magnification than that of Fig. 4 to demonstrate the migrating epidermal cells dissecting between the desiccated clot and wound base. The section has been hybridized with FN-C and viewed with brightfield (*A*) and darkfield (*B*) optics as in Fig. 3. Note that the section shows the opposite edge of a wound from that in Figs. 3 and 4, and the direction of migration is therefore left to right. Intense labeling is seen in the granulation tissue (*GT*) under the migrating epidermal cells (*EP*), and also in the immediately adjacent dermal cells (*DE*). Note, however, that the epidermal cells themselves are unlabeled (*asterisks*). The low level of labeling seen above the epidermal cells represents nonspecific binding of the FN-C probe to the clot covering the wound, and was also observed in the sense controls (not shown). Bar, 50 μm.
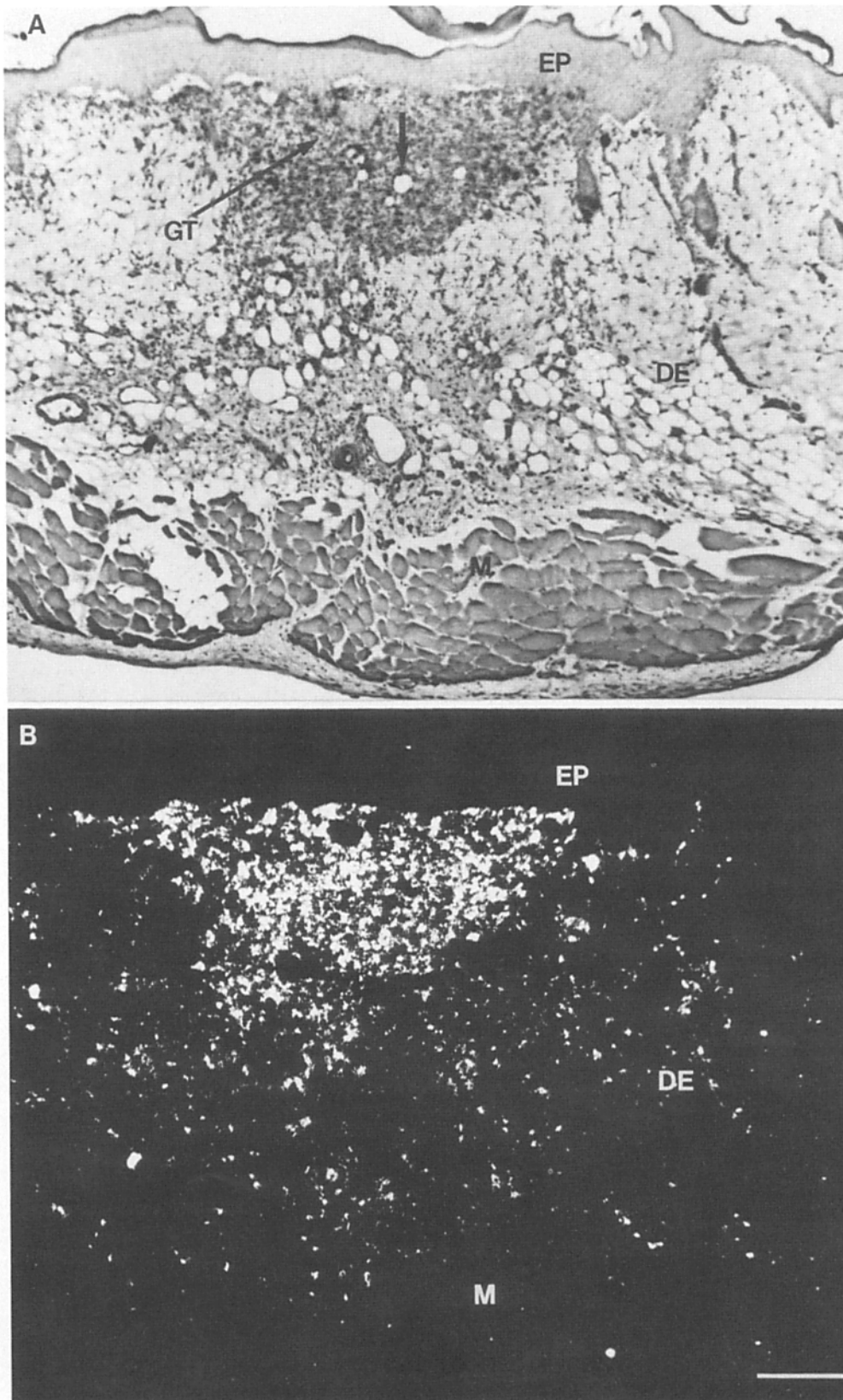
Figure 6. A section through an entire day 7 wound hybridized and viewed as in Figs. 3 and 4. The smaller wound area than that seen at 4 d reflects wound contraction between the two times. The granulation tissue (*GT*) (blood vessel indicated by a *bold arrow*) remains intensely labeled. Labeled cells in the muscle (*M*) and dermis (*DE*) are less numerous than at 2 and 4 d. Note that the epidermis (*EP*) now completely covers the wound and remains unlabeled. Bar, 200 μm.
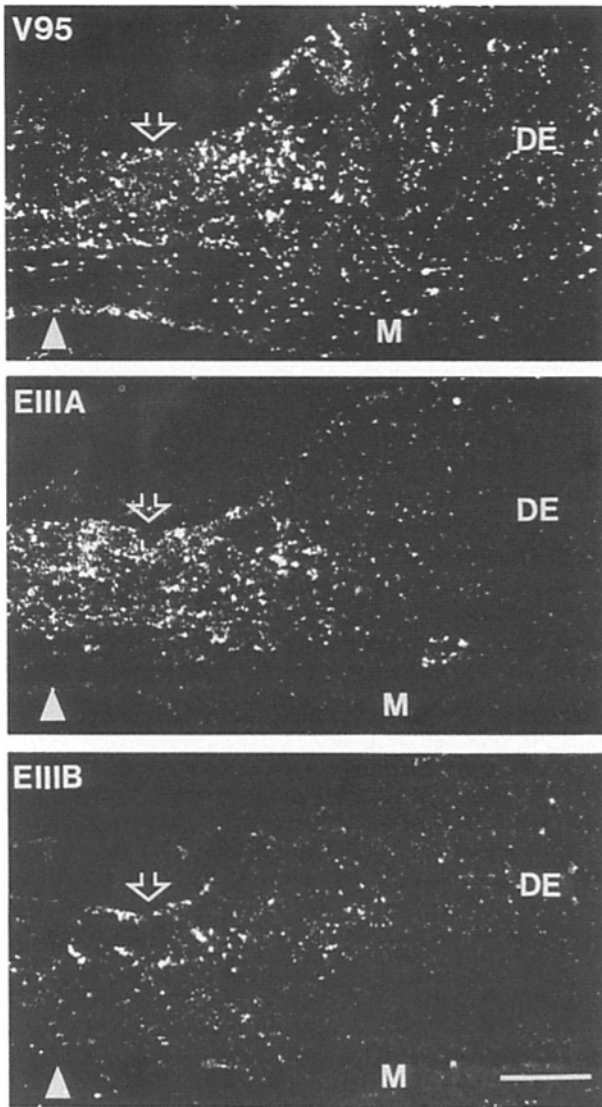
specific activity and concentration, these data suggest that EIIIB⁺ forms of FN mRNA are less abundant than EIIIA⁺ forms in the area of the wound.

No specific labeling was seen when the sections of wounded skin were hybridized with sense probes synthesized from the FN-EIIIA, FN-EIIIB, and FN-V constructs, confirming the specificity of these segment-specific probes (data not shown).

The observed restriction of expression of EIIIA and EIIIB in FN mRNA after wounding could result either from a splicing difference or from an overall increase in the level of all variants of FN mRNA so that a previous undetectable level of EIIIA⁺ or EIIIB⁺ FN mRNA was now sufficient to produce a hybridization signal. To confirm the appearance of a change in alternative splicing we hybridized adjacent sections of a 4-d wound with FN-C, FN-EIIIA, or FN-EIIIB, and then exposed the sections to autoradiographic emulsion for different periods of time such that the level of labeling in the wound bed and edges was as great or greater with FN-EIIIA or FN-EIIIB than with FN-C (Fig. 8). If the cells in the adjacent dermis and muscle contain the same proportion of EIIIA and EIIIB in their FN mRNA as do those at the wound base and edges, they should now also label as, or

Figure 7. Three closely adjacent sections through the edge of the same 4-d wound as that shown in Fig. 4, illustrating the appearance of EIIIA⁺ and EIIIB⁺ FN mRNA after wounding. The sections were hybridized with the segment-specific probes FN-V95, FN-EIIIA, and FN-EIIIB as indicated and viewed with darkfield optics. FN-V95 labels in a manner identical to FN-C (shown in Fig. 4), with intense labeling both of the wound base and edge (*hollow arrow*) as well as of adjacent dermis (*DE*) and muscle (*M*). FN-EIIIA also labels the base and edges of the wound, but shows a more restricted distribution with little labeling of the muscle (*M*) and of the dermis (*DE*). FN-EIIIB labels the same areas as FN-EIIIA but with a lower intensity. Note that the connective tissue layer under the muscle (*arrowheads*) remains A⁻B⁻V⁺ after wounding. Bar, 500 μm.
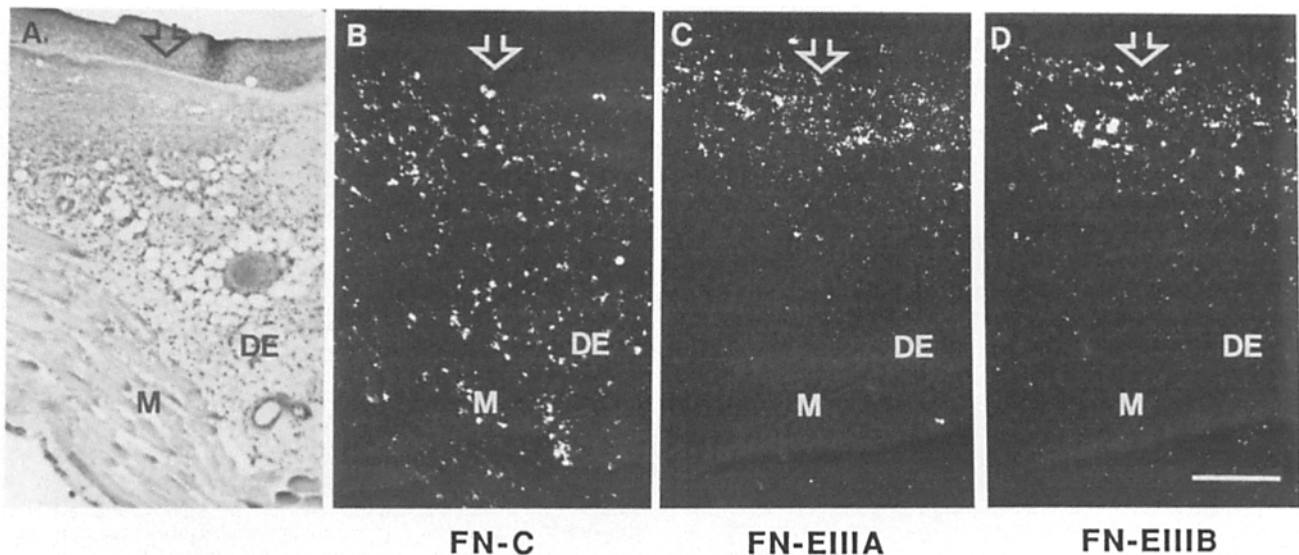
Figure 8. Three closely adjacent sections through the base of a 4-d wound hybridized with FN-C, FN-EIIIA, and FN-EIIIB, exposed for 1, 2, and 7 d, respectively, and then viewed with darkfield optics (*B–D*). A representative section is shown in brightfield optics (*A*). As explained in the text, the absence of labeling in the dermis (*DE*) and muscle (*M*) with FN-EIIIA and FN-EIIIB in the presence of more intense labeling in the base of the wound (*hollow arrow*) with these probes than with the FN-C probe shows that the restricted expression of EIIIA and EIIIB after wounding reflects a difference in RNA splicing between the two regions. Bar, 300 μm.

more, intensely with FN-EIIIA or FN-EIIIB as with FN-C. However, in these experiments the restricted distribution of EIIIA and EIIIB was still seen, with little labeling outside the base and edges of the wound (Fig. 8). We conclude, therefore, that the observed pattern of labeling with the splice segment–specific probes reflects a splicing difference in which EIIIA and EIIIB are included in the FN mRNA at the base and sides of the wound to a greater degree than they are in normal skin or in cells more distant from the wound.

Discussion

To determine the patterns of FN gene expression and splicing in healing wounds, we performed in situ hybridization on tissue sections of normal rat skin and wounds at different times during healing, using probes that recognize either total FN mRNA or mRNA containing the individual spliced segments. We found a low but significant level of FN mRNA in normal skin localized to scattered individual cells in the reticular dermis and to cells adjacent to and within the panniculus carnosus. In wounded skin, by contrast, extensive expression of FN mRNA was found at the base and edges of the wound, extending into the adjacent uninjured dermis and to the underlying muscle. This increased level of FN mRNA was evident within 1 d after wounding, with the highest levels seen at the fourth and seventh day. Analysis of the splicing pattern showed that this FN mRNA was spatially heterogeneous. Cells at the base and edges of the wound expressed mRNA that included both of the alternatively spliced EIIIA and EIIIB regions; in contrast, mRNA expression in adjacent dermis and muscle showed the same splicing pattern as that seen in normal skin; that is, EIIIA and EIIIB were largely excluded. Thus, (a) wounding induces increased levels of FN mRNA both directly beneath the wounded area and in adjacent dermal tissue; and (b) the pattern of splicing of FN mRNA locally in the wound bed, but not that in contiguous but more distant sites, differs from that in normal skin.

These results are consistent with previous immunolocalization studies of FN in normal and wounded skin. Studies in normal rat skin have demonstrated FN surrounding hair follicles in the dermis (Couchman et al., 1979) and reported either a low or undetectable level of FN in the normal epidermal basement membrane (Couchman et al., 1979; Clark et al., 1983). After wounding, FN levels are reported to rise substantially at the base and edges of the wound (Grinnell et al., 1981; Clark et al., 1982a, b). At least some of this FN is apparently synthesized by cells in the immediate vicinity of the wound, as studies using mice in which well-healed transplants of rat skin were wounded have shown that locally synthesized (rat) FN is present in the zone of healing (Clark et al., 1983). These studies also showed that rat FN was less abundant than the mouse pFN for the first 4 d after wounding (Clark et al., 1983). However, our results show a dramatic early increase in FN mRNA at the base of the wound, and in the dermis forming the edge of the wound. This suggests that locally produced FN is present from the earliest stages of healing. Moreover, this locally produced FN contains spliced segments not present in the pFN that constitutes the majority form of FN at these early stages. We hypothesize that these new forms of FN may perform qualitatively different functions.

A central event in healing skin wounds is the inward migration of epidermal cells from the wound edges to cover the injured surface. As judged by immunohistochemistry, these migrating cells are underlain by an irregular band of fibrin and FN (Clark et al., 1982a; Clark et al., 1983; Dvorak et al., 1984). This is in contrast to the situation in normal, uninjured skin where the epidermal basal lamina contains little or no fibrin or FN (Couchman et al., 1979). The source of the FN associated with migrating keratinocytes has not been established, and it could be derived from plasma or from local synthesis by epidermal or dermal cells.

A striking feature observed in our study was the expression of FN by cells disposed at the base and edges of the wound just beneath the epidermis; this FN mRNA expression preceded the inward migration of epidermal cells that eventually covered the wound. FN-coated glass coverslips inserted under the skin of newts are reported to promote epidermal cell migration (Donaldson and Mahan, 1983). Epidermal cells also express a 140-kD FN receptor similar to fibroblast FN receptors (Toda et al., 1987; Grinnell et al., 1987). It seems likely that expression of this epidermal cell receptor is increased after wounding, as these cells show increased adhesion to FN while migrating over a wound base (Takashima et al., 1986) and the Arg-Gly-Asp-Ser (RGDS) peptide, which inhibits the binding of FN by the integrin receptors (Ruoslahti and Pierschbacher, 1987) can prevent epidermal cell migration after wounding (Donaldson et al., 1987). Together, these findings suggest that the cells containing FN mRNA at the wound edges synthesize a matrix which forms a pathway of FN. This pathway may then be followed by migrating epidermal cells expressing integrin receptors that bind this FN. Our observation that neither migrating nor normal epidermal cells express significant levels of FN mRNA suggests that they are not responsible for the synthesis of the FN over which they migrate. Instead, without competition by endogenous FN, receptors on epidermal cells may then be maximally responsive to FN encountered within the pathway.

Our finding that epidermal cells migrating over the wound bed contain little or no FN mRNA in vivo is in contrast with in vitro studies, in which these cells synthesize FN (Alitalo et al., 1982; Clark et al., 1985; Kariniemi et al., 1982; O'Keefe et al., 1984, 1987; Kubo et al., 1987), and illustrates the caution required when extrapolating from in vitro models of wound healing to the in vivo situation. It is important, therefore, to establish the source(s) of FN in vivo. Although keratinocytes do not appear to express FN mRNA at any time, present methods have not yet permitted us to determine the respective roles played by individual inflammatory cells or dermal fibroblasts in producing the various types of FN synthesized during repair. Healing wounds are infiltrated by a heterogeneous population of inflammatory cells as well as by activated connective tissue cells. Methods for improving the preservation of tissue morphology to extend positive cell identification are currently under development.

The demonstration that the pattern of FN splicing in the area of the healing wound is different from that in normal skin is a major finding of this study. In normal skin all FN mRNA appears to be EIIIA⁻, EIIIB⁻, but in wounds we found EIIIA⁺ and/or EIIIB⁺ FN mRNA localized to the wound base and edges at all stages after wounding. This is particularly interesting in light of our recent demonstration

that both EIIIA and EIIIB are included in the majority of the FN mRNA present in the early (embryonic days 2–4) chicken embryo (ffrench-Constant and Hynes, 1988), when there is active proliferation and migration of cells. However, the EIIIA and EIIIB regions are spliced out in tissue-specific patterns after embryogenesis and organogenesis have been largely completed (ffrench-Constant and Hynes, 1989). Although we have not examined embryonic rat FN mRNA directly, the patterns of FN splicing appear to be highly conserved among species; for example, hepatocytes synthesizing pFN and fibroblasts synthesizing cellular FN in culture show identical splicing patterns in chickens, rats, and humans (Schwarzbauer et al., 1983, 1987; Kornblihtt et al., 1985; Sekiguchi et al., 1986; Norton and Hynes, 1987). It therefore seems likely that the early rat embryo, like the chicken, contains predominantly EIIIA$^+$, EIIIB$^+$ FN mRNA and that the results observed in this study reflect a reappearance of an embryonic pattern of FN splicing. Previous observations show that the pattern of FN mRNA splicing in cultured cells is accurately reflected in the FN synthesized by these cells (Tamkun and Hynes, 1983; Price and Hynes, 1985; Paul et al., 1986; Schwarzbauer et al., 1987). Therefore, our study provides evidence that splicing of FN mRNA during wound healing leads to different, and possibly functionally more appropriate, forms of FN than are present either from plasma or from nearby cells that do not alter their pattern of splicing from that seen in normal skin.

Although these results do not address the actual function(s) of the EIIIA$^+$, EIIIB$^+$ FN, the localization of this form of FN at the base and sides of the wound is consistent with a role in epithelial cell migration. In keeping with this, EIIIA$^+$, EIIIB$^+$ FN is widespread in the embryo at a time when many different cell migrations are in progress (Norton and Hynes, 1987; ffrench-Constant and Hynes, 1988). However, other functions for this form of FN such as a role in cell proliferation and differentiation or in chemotaxis obviously should not be excluded.

The mechanisms responsible for controlling the pattern of splicing change are unknown. However, TGF-β, which increases the synthesis of both FN and its integrin receptor (Ignotz et al., 1987; Ignotz and Massague, 1987; Raghow et al., 1987; Roberts et al., 1988), has been shown by analyses of FN protein to increase the inclusion of EIIIA in FN (Balza et al., 1988). This growth factor has been proposed to play an important role in wound healing, as the subcutaneous injection of TGF-β produces a cellular response which mimics the formation of granulation tissue found during wound healing (Roberts et al., 1986). TGF-β is found in high concentrations in platelets (Assoian et al., 1983) and would therefore be expected to be released at the site of wounding, and TGF-β mRNA has also been demonstrated in the macrophages present in a wound (Rappolee et al., 1988). It seems likely, therefore, that this and other growth factors will be involved in the molecular and cellular mechanisms of healing and further in vitro studies of their role in the FN RNA splicing associated with wound healing are clearly warranted.

The finding that all detectable FN mRNA in normal skin was EIIIA$^-$, EIIIB$^-$ was unexpected, as this pattern of FN splicing has been considered a characteristic of pFN (Hynes, 1985; Schwarzbauer et al., 1987; Gutman and Kornblihtt, 1987; Zardi et al., 1987). Given the correlation between these in situ hybridization results and the pattern of FN labeling using immunolocalization, it seems likely that EIIIA$^-$, EIIIB$^-$ FN is synthesized in normal adult skin and deposited in the local matrix. This result raises the possibility that splicing of the V region, rather than of EIIIA or EIIIB, may represent an important difference between soluble pFN, synthesized in the liver, and locally produced "cellular" FN. V is included in only 50% of pFN mRNA (Tamkun and Hynes, 1983; Schwarzbauer et al., 1983, 1987; Paul and Hynes, 1984; Paul et al., 1986; Norton and Hynes, 1987), whereas it appears to be present in the great majority of the FN mRNA present in embryos (Norton and Hynes, 1987; ffrench-Constant and Hynes, 1988). In addition, our quantitative study of FN mRNA in 16-d chicken embryo using ribonuclease protection experiments found apparently complete V region inclusion in all tissues examined except the liver, which synthesizes pFN (ffrench-Constant and Hynes, 1989). In this study, the level of inclusion of V95 appears to be high, as judged by labeling intensity (Figs. 2 and 7), although more quantitative analyses such as nuclease protection are needed to strengthen this conclusion. Further studies of V region splicing, and in particular of the V25 region which was not examined in our study but which can be alternatively spliced in hepatocytes and fibroblasts (Paul et al., 1986), are required to determine the contribution of this region to the properties of FN.

The synthesis of EIIIA$^+$, EIIIB$^+$ "embryonic-type" FN after wounding suggests that the EIIIA$^-$, EIIIB$^-$ pFN, which is present as a result of exudation from damaged vessels and which represents the most abundant form of FN until at least the fourth day after wounding (Clark et al., 1983), may not be sufficient for satisfactory healing. This point has therapeutic implications, as a number of clinical trials have attempted to accelerate wound healing using exogenously applied pFN (Clark, 1988; Grinnell et al., 1988). In light of our results, it seems likely that FN containing both EIIIA and EIIIB might provide the most efficient acceleration of wound repair.

References

Ali, I. U., and R. O. Hynes. 1978. Effect of LETS glycoprotein on cell motility. *Cell.* 14:439–446.

Alitalo, K., E. Kuismanen, R. Myllyla, V. Kiistala, S. Asko-Seljavaana, and A. Vaheri. 1982. Extracellular matrix proteins of human epidermal keratinocytes and feeder 3T3 cells. *J. Cell Biol.* 94:497–505.

Angerer, L. M., K. H. Cox, and R. C. Angerer. 1987. Demonstration of tissue-specific gene expression by in situ hybridization. *Methods Enzymol.* 152:649–661.

Assoian, R. K., A. Komoriya, C. A. Meyers, D. M. Miller, and M. B. Sporn. 1983. Transforming growth factor β in human platelets. Identification of a major storage site, purification and characterization. *J. Biol. Chem.* 258:7155–7160.

Balza, E., L. Borsi, G. Allemanni, and L. Zardi. 1988. Transforming growth factor β regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 228:42–44.

Berger, C. N. 1986. In situ hybridization of immunoglobulin-specific RNA in single cells of the B lymphocyte lineage with radiolabelled DNA probes. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:85–93.

Boucaut, J. C., T. Darriebere, H. Boulekbache, and J. P. Thiery. 1984. Prevention of gastrulation but not neuralation by antibodies to fibronectin in amphibian embryos. *Nature (Lond.).* 307:364–367.

Bowersox, J. C., and N. Sorgente. 1982. Chemotaxis of aortic endothelial cells in response to fibronectin. *Cancer Res.* 42:2547–2551.

Bronner-Fraser, M. 1985. Alterations in neural crest migration by a monoclonal antibody that affects cell adhesion. *J. Cell Biol.* 101:610–617.

Bronner-Fraser, M. 1986. An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development in vivo. *Dev. Biol.* 117:528–536.

Clark, R. A. 1988. Potential roles of fibronectin in cutaneous wound repair. *Arch. Dermatol.* 124:201–206.

Clark, R. A., L. D. Nielsen, S. E. Howell, and J. M. Folkvord. 1985. Human keratinocytes that have not terminally differentiated synthesize laminin and fibronectin but deposit only fibronectin in the pericellular matrix. *J. Cell. Biochem.* 28:127–141.

Clark, R. A. F., and P. M. Henson. 1988. The Molecular and Cellular Biology of Wound Repair. Plenum Press, New York. 597 pp.

Clark, R. A. F., P. DellaPelle, E. Manseau, J. M. Lanigan, H. F. Dvorak, and R. B. Colvin. 1982b. Blood vessel fibronectin increases in conjunction with endothelial cell proliferation and capillary ingrowth during wound healing. *J. Invest. Dermatol.* 79:269–276.

Clark, R. A. F., J. M. Lanigan, P. DellaPelle, E. Manseau, H. F. Dvorak, and R. B. Colvin. 1982a. Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization. *J. Invest. Dermatol.* 79:264–269.

Clark, R. A. F., N. E. Wilkner, D. E. Doherty, and D. A. Norris. 1988. Cryptic chemotactic activity of fibronectin for human monocytes resides in the 120-kDa fibroblastic cell-binding fragment. *J. Biol. Chem.* 263:12115–12123.

Clark, R. A. F., H. J. Winn, H. F. Dvorak, and R. B. Colvin. 1983. Fibronectin beneath reepithelializing epidermis in vivo: sources and significance. *J. Invest. Dermatol.* 80:26s–30s.

Couchman, J. R., W. T. Gibson, D. Thom, A. C. Weaver, D. A. Rees, and W. E. Parish. 1979. Fibronectin distribution in epithelial and associated tissues in the rat. *Arch. Dermatol. Rev.* 266:295–310.

Critchley, D. R., M. A. England, J. Wakely, and R. O. Hynes. 1979. Distribution of fibronectin in the ectoderm of gastrulating chick embryos. *Nature (Lond.).* 280:498–500.

Donaldson, D. J., and J. T. Mahan. 1983. Fibrinogen and fibronectin as substrates for epidermal cell migration during wound closure. *J. Cell Sci.* 62:117–127.

Donaldson, D. J., J. T. Mahan, and G. N. Smith, Jr. 1987. Newt epidermal cell migration in vivo and in vitro appears to involve Arg-Gly-Asp-Ser receptors. *J. Cell Sci.* 87:525–534.

Duband, J. L., and J. P. Thiery. 1982. Distribution of fibronectin in the early phase of avian cephalic neural crest cell migration. *Dev. Biol.* 93:308–323.

Dufour, S., J.-L. Duband, A. R. Kornblihtt, and J. P. Thiery. 1988. The role of fibronectins in embryonic cell migrations. *Trends Genet.* 4:198–203.

Dvorak, H. F., D. M. Form, E. J. Manseau, and B. D. Smith. 1984. Pathogenesis of desmoplasia. I. Immunofluorescence identification and localization of some structural proteins of line 1 and line 10 guinea pig tumors and of healing wounds. *J. Natl. Cancer. Inst.* 73:1195–1205.

ffrench-Constant, C., and R. O. Hynes. 1988. Patterns of fibronectin gene expression and splicing during cell migration in chicken embryos. *Development.* 104:369–382.

ffrench-Constant, C., and R. O. Hynes. 1989. Alternative splicing of fibronectin is temporally and spatially regulated in the chicken embryo. *Development.* 106:375–388.

Grinnell, F., K. I. Toda, and A. Takashima. 1987. Activation of keratinocyte fibronectin receptor function during cutaneous wound healing. *J. Cell Sci.* 8(Suppl.):199–209.

Grinnell, F., K. Toda, and A. Takashima. 1988. Role of fibronectin in epithelialization and wound healing. *Prog. Clin. Biol. Res.* 266:259–272.

Grinnell, F., R. E. Billingham, and L. Burgess. 1981. Distribution of fibronectin during wound healing in vivo. *J. Invest. Dermatol.* 76:181–189.

Gutman, A., and A. R. Kornblihtt. 1987. Identification of a third region of cell-specific alternative splicing in human fibronectin mRNA. *Proc. Natl. Acad. Sci. USA.* 84:7179–7182.

Heasman, J., R. O. Hynes, A. P. Swan, V. Thomas, and C. C. Wylie. 1981. Primordial germ cells of *Xenopus* embryos: the role of fibronectin in their adhesion during migration. *Cell.* 27:437–447.

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

Humphries, M. J., S. K. Akiyama, A. Komoriya, K. Olden, and K. M. Yamada. 1988. Neurite extension of chicken peripheral nervous system neurons on fibronectin: relative importance of specific adhesion sites in the central cell-binding domain and the alternatively spliced type III connecting segment. *J. Cell Biol.* 106:1289–1297.

Humphries, M. J., A. Komoriya, S. K. Akiyama, K. Olden, and K. M. Yamada. 1986. Identification of alternatively spliced site in human plasma

fibronectin that mediates cell type-specific adhesion. *J. Cell Biol.* 103:2637–2647.

Hynes, R. O. 1985. Molecular biology of fibronectin. *Annu. Rev. Cell Biol.* 1:67–90.

Ignotz, R. A., T. Endo, and J. Massague. 1987. Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-β. *J. Biol. Chem.* 262:6443–6446.

Ignotz, R. A., and J. Massague. 1987. Cell adhesion protein receptors as targets for transforming growth factor-β action. *Cell.* 51:189–197.

Kariniemi, A.-L., V.-P. Lehto, T. Vartio, and I. Virtanen. 1982. Cytoskeleton and pericellular matrix organization of pure adult human keratinocytes cultured from suction-blister root epidermis. *J. Cell Sci.* 58:49–61.

Kitten, G. T., R. R. Markwald, and D. L. Bolender. 1987. Distribution of basement membrane antigens in cryopreserved early embryonic hearts. *Anat. Rec.* 217:379–390.

Kornblihtt, A. R., K. Umezawa, K. Vibe-Pedersen, and F. E. Baralle. 1985. Primary structure of human plasma fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1755–1759.

Kubo, M., M. Kan, M. Isemura, I. Yamane, and H. Tagami. 1987. Effects of extracellular matrices on human keratinocyte adhesion and growth and on its secretion and deposition of fibronectin in culture. *J. Invest. Dermatol.* 88:594–601.

Kurkinen, M., K. Alitalo, A. Vaheri, S. Stenman, and L. Saxen. 1979. Fibronectin in the development of embryonic chick eye. *Dev. Biol.* 69:589–600.

Kurkinen, M., A. Vaheri, P. J. Roberts, and S. Stenman. 1980. Sequential appearance of fibronectin and collagen in experimental granulation tissue. *Lab. Invest.* 43:47–51.

Linask, K. K., and J. W. Lash. 1986. Precardiac cell migration: fibronectin localization at mesoderm-endoderm interface during directional movement. *Dev. Biol.* 114:87–101.

Mayer, B. W., E. D. Hay, and R. O. Hynes. 1981. Immunocytochemical localization of fibronectin in embryonic chick trunk and area vasculosa. *Dev. Biol.* 82:267–286.

Newgreen, D., and J. P. Thiery. 1980. Fibronectin in early avian embryos: synthesis and distribution along the migration pathways of neural crest cells. *Cell Tissue Res.* 211:269–291.

Norris, D. A., R. A. F. Clark, L. M. Swigart, J. C. Huff, W. L. Weston, and S. E. Howell. 1982. Fibronectin fragment(s) are chemotactic for human peripheral blood monocytes. *J. Immunol.* 129:1612–1618.

Norton, P. A., and R. O. Hynes. 1987. Alternative splicing of chicken fibronectin in embryos and in normal and transformed cells. *Mol. Cell. Biol.* 7:4297–4307.

O'Keefe, E. J., D. T. Woodley, R. J. Falk, W. R. Gammon, and R. A. Briggaman. 1987. Production of fibronectin by epithelium in a skin equivalent. *J. Invest. Dermatol.* 88:634–639.

O'Keefe, E. J., D. T. Woodley, G. Castillo, N. Russell, and R. E. Payne. 1984. Production of soluble and cell-associated fibronectin by cultured keratinocytes. *J. Invest. Dermatol.* 82:150–55.

Paul, J., and R. O. Hynes. 1984. Multiple fibronectin subunits and their posttranslational modifications. *J. Biol. Chem.* 259:13407–13487.

Paul, J. I., J. E. Schwarzbauer, J. W. Tamkun, and R. O. Hynes. 1986. Cell-type-specific fibronectin subunits generated by alternative splicing. *J. Biol. Chem.* 261:12258–12265.

Poole, T. J., and J. P. Thiery. 1986. Antibodies and a synthetic peptide that block cell-fibronectin adhesion arrest neural crest cell migration in vivo. *Prog. Clin. Biol. Res.* 217B:235–238.

Price, J., and R. O. Hynes. 1985. Astrocytes in culture synthesize and secrete a variant form of fibronectin. *J. Neurosci.* 5:2205–2211.

Raghow, R., A. E. Postlethwaite, J. Keski-Oja, H. L. Moses, and A. H. Kang. 1987. Transforming growth factor-β increases steady state levels of type 1 procollagen and fibronectin messenger RNAs posttranscriptionally in cultured human dermal fibroblasts. *J. Biol. Chem.* 263:4586–4592.

Rappolee, D. A., D. Mark, M. J. Banda, and Z. Werb. 1988. Wound macrophages express TGF-α and other growth factors in vivo: analysis by mRNA phenotyping. *Science (Wash. DC).* 241:708–712.

Roberts, C. J., T. M. Birkenmeier, J. J. McQuillan, S. K. Akiyama, S. S. Yamada, W. T. Chen, K. M. Yamada, and J. A. McDonald. 1988. Transforming growth factor β stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts. *J. Biol. Chem.* 263:4586–4592.

Roberts, A. B., M. B. Sporn, R. K. Assoian, J. M. Smith, N. S. Roche, L. M. Wakefield, U. I. Heine, L. A. Liotta, V. Falanga, J. H. Kehrl, and A. S. Fauci. 1986. Transforming growth factor type β: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc. Natl. Acad. Sci. USA.* 83:4167–4171.

Rovasio, R. A., A. Delouvee, K. M. Yamada, R. Timpl, and J. P. Thiery. 1983. Neural crest cell migration: requirements for exogenous fibronectin and high cell density. *J. Cell Biol.* 96:462–473.

Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science (Wash. DC).* 238:491–497.

Schwarzbauer, J. E., R. E. Patel, D. Fonda, and R. O. Hynes. 1987. Multiple sites of alternative splicing in the rat fibronectin gene transcript. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2573–2580.

Schwarzbauer, J. E., J. W. Tamkun, I. R. Lemischka, and R. O. Hynes. 1983.

Three different fibronectin mRNAs arise by alternative splicing within the coding region. *Cell.* 35:421–431.

Sekiguchi, K., A. M. Klos, K. Kurachi, S. Yoshitake, and S. Hakomori. 1986. Human liver fibronectin complementary DNAs: identification of two different messenger RNAs possibly encoding the α and β subunits of plasma fibronectin. *Biochemistry.* 25:4936–4941.

Takashima, A., R. E. Billingham, and F. Grinnell. 1986. Activation of rabbit keratinocyte receptor function in vivo during wound healing. *J. Invest. Dermatol.* 86:585–590.

Tamkun, J. W., and R. O. Hynes. 1983. Plasma fibronectin is synthesised and secreted by hepatocytes. *J. Biol. Chem.* 258:4641–4647.

Thiery, J. P., J. L. Duband, and A. Delouvee. 1982. Pathways and mechanisms of avian trunk neural crest cell migration and localization. *Dev. Biol.* 93:324–343.

Toda, K., and F. Grinnell. 1987. Activation of human keratinocyte receptor function in relation to other ligand-receptor interactions. *J. Invest. Dermatol.* 88:412–417.

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