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Patterns of fibronectin gene expression and splicing during cell migration in chicken embryos

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Summary

A variety of evidence suggests that fibronectin (FN) promotes cell *migration* during embryogenesis, and it has been suggested that the deposition of FN along migratory pathways may also play a role in cell *guidance*. In order to investigate such a role for FN, it is important to determine the relative contribution of migrating and pathway-forming cells to the FN in the migratory track, as any synthesis of FN by the migrating cells might be expected to mask guidance cues provided by the exogenous FN from pathway-forming cells. We have therefore used *in situ* hybridization to determine in developing chicken embryos the distribution and alternative splicing of FN mRNA during three different cell migrations known to occur through FN-rich environments; neural crest cell migration, mesenchymal cell migration in the area vasculosa and endocardial cushion cell migration in the heart. Our results show that trunk neural crest cells do not contain significant FN mRNA during their initial migration. In contrast, migrating mesenchymal cells of the area vasculosa and endocardial cushion cells both contain abundant FN mRNA. Furthermore, the FN mRNA in these migrating mesenchymal and endocardial cells appears to be spliced in a manner identical with that present in the cells adjacent to their pathways. This *in vivo* evidence for FN synthesis by migrating and pathway cells argues against a generalized role for exogenously produced FN as a guidance mechanism for cell migration.

Key words: fibronectin, alternative splicing, cell migration, neural crest, area vasculosa, heart, *in situ* hybridization, chicken embryogenesis.

Introduction

The migration of cells along specific pathways throughout the developing embryo represents an important part of morphogenesis. The cellular and molecular mechanisms by which this migration is controlled remain poorly understood. However, a number of experimental observations suggest that the interaction of cells with fibronectin (FN) *via* specific cell-surface receptor(s) plays an important role. FN promotes cell migration *in vitro* (Ali & Hynes, 1978; Rovasio *et al.* 1983) and is present in the extracellular matrix through which many different embryonic cell types migrate including gastrulating mesodermal cells (Critchley *et al.* 1979), neural crest cells (Newgreen & Thiery, 1980), mesenchymal cells in the area vasculosa (Mayer *et al.* 1981), corneal cells (Kurkinen *et al.* 1979), primordial germ cells (Heasman *et al.* 1981) precardiac cells (Linask & Lash, 1986) and endocardial cushion cells (Kitten *et al.* 1987). At least in the case of avian cranial neural crest cell migration and amphibian and insect gastrulation, antibodies against fibronectin will block cell migration *in vivo* (Poole & Thiery, 1986; Boucaut *et al.* 1984; Gratecos *et al.* 1988). More recently, it has been shown that migrating neural crest cells express receptors of the integrin family (Duband *et al.* 1986; Krotoski *et al.* 1986), which includes at least one FN receptor (see Hynes (1987) for review), and that antibodies against the integrin complex will also block migration *in vivo* (Bronner-Fraser, 1985, 1986).

In addition to promoting cell *migration*, FN may also play a role in the *guidance* of the cells along their migratory pathways. Immunocytochemical studies examining the synthesis of FN *in vitro* by migrating cells and by cells adjacent to their migration pathways have shown that almost all trunk neural crest cells as well as primordial germ cells do not synthesize FN *in*

vitro, while those cells adjacent to their pathway *in vivo* do synthesize FN *in vitro* (Newgreen & Thiery, 1980; Heasman *et al.* 1981). Based on these observations, a simple model has been proposed in which the lack of FN synthesis by migrating cells increases their sensitivity to exogenous FN produced by pathway-forming cells, which could therefore provide cues to guide migration (Newgreen & Thiery, 1980). Tracks of exogenous FN produced by these pathway-forming cells could also specify the direction of migration; during development of the heart, both the precardiac cells and the endocardial cushion cells appear to migrate along gradients of FN (Linask & Lash, 1986; Kitten *et al.* 1987; Mjaatvedt *et al.* 1987), raising the possibility that the direction taken by these migrating cells results from their haptotaxis towards an increasing concentration of FN.

The role of exogenous FN in the guidance of cell migration suggested by these results emphasizes the need to determine directly *in vivo* whether migrating cells and/or cells adjacent to their pathway synthesize the FN present in the migratory track, as any synthesis of FN by the migrating cells might be expected to mask guidance cues provided by the exogenous FN from pathway-forming cells. Moreover, it is also necessary to determine the precise type of FN produced by different cell types associated with cell migration. Recent work on the molecular biology of FN has demonstrated that FN heterogeneity is generated *in vivo* by alternative splicing of the primary FN gene transcript so as to include or exclude all or part of three separate exons, each of which is derived from one of the so-called type III repeats that constitute the central part of the molecule (Fig. 1) (Hynes, 1985). In rats, humans and chickens, two of these exons (designated EIIIB and EIIIA) are either completely included or excluded, while the third exon (designated the variable or V region) can be partially excluded (Fig. 1) (Hynes, 1985; Kornblihtt *et al.* 1985; Paul *et al.* 1986; Schwarzbauer *et al.* 1987; Norton & Hynes, 1987). The V region of human FN has been shown to contain cell binding sites (Humphries *et al.* 1986, 1987), and splicing changes may therefore produce forms of FN with differing adhesive properties that may play different roles in the promotion and/or guidance of cell migration. For example, the synthesis and secretion of FN by migrating cells need not diminish the cells' sensitivity to exogenous FN if the two forms of FN are different. Equally, the distribution of different types of exogenous FN within the extracellular matrix may represent a mechanism for pathway specification.

In this paper, we have examined the pattern of FN synthesis in migrating cells and in cells adjacent to their pathways by studying the distribution and alternative splicing of FN mRNA *in vivo* by *in situ* hybridization. We show that migrating trunk neural crest cells contain little or no FN mRNA, consistent with the previous immunocytochemical results of Newgreen & Thiery (1980). In contrast, however, to these results in trunk neural crest cells, we show that migrating mesenchymal cells in the area vasculosa and endocardial cushion cells of the heart both contain abundant FN mRNA. Furthermore, this FN mRNA appears to be spliced in a pattern identical with that present in the cells adjacent to the pathway, suggesting that these migratory cells contribute additional FN to their migratory substrate indistinguishable from that already deposited by the pathway-forming cells. These contrasting distributions of FN mRNA in three different cell migrations suggest that the absence of FN synthesis by migrating cells is not a consistent feature of cell migration within FN-rich pathways, and that the proposed mechanism by which exogenous FN plays a role in trunk neural crest cell guidance cannot therefore be generalized to all other cell migrations.

Materials and methods

Preparation of tissue

Fertilized eggs (Spafas, CT) were kept in a humidified incubator for appropriate times, after which the embryos were removed, staged according to Hamburger & Hamilton (1951) and fixed in ice-cold 5% acetic acid, 4% formaldehyde and 85% ethanol (AFE). Stage-10 to -24 (day-2 to -4) embryos were fixed by immersion for 1 h. For the experiments examining embryonic day-17 liver, the embryos were decapitated, perfused through the ascending aorta with cold AFE after which the liver was removed, cut into 2 mm slices and immersed in AFE for a further 2–3 h. After fixation, the different tissues were washed in 95% ethanol, dehydrated and embedded in wax (Paraplast Xtra, Polysciences Inc.). Sections were cut at 5–7 μm, placed onto slides coated by immersion in a 50 $\mu g\,ml^{-1}$ solution of poly-L-lysine and left on a warm plate (45°C) overnight before use.

Preparation of probes

Single-stranded RNA probes were generated by transcription from short cDNA sequences which had been isolated from appropriate restriction enzyme digests of a larger chicken cDNA clone (Norton & Hynes, 1987; kindly provided by Dr P. A. Norton) and ligated into the polylinker sequence between the SP6 and T7 RNA polymerase sites of pGEM plasmids (Promega Biotec). Four such constructs were used in this study. The first (designated FN-C) contained a 160 nt sequence of FN cDNA coding for part of the type-1 repeats at the C-terminal end of the molecule (see Fig. 1) and corresponding to a region of mRNA always included in the spliced message. The three other constructs contained cDNA corresponding to parts of the variably spliced regions. The cDNA fragments in the EIIIA and EIIIB constructs (FN-EIIIA and FN-EIIIB, of 160 nt and

230 nt, respectively) contained sequences entirely within the two spliced regions of the primary gene transcript. The V region cDNA construct (FN-V) of 213 nt included 69 nt from the adjacent 5′ unspliced region, but was used to generate V-region-specific probes as explained below. The identity and orientation of the inserts was confirmed by sequencing.

Plasmids were linearized at one or other end of the polylinker and RNA probes, which were labelled with ^{35}S-UTP (NEN) to a specific activity of approximately 10^8 cts min^{-1} μg^{-1}, were synthesized using SP6 or T7 RNA polymerase and the reagents supplied in a commercially available transcription kit (Promega Biotec). Antisense and sense probes were synthesized from all constructs except FN-V, which was linearized within the insert at the *Sau*3A site at the 5′ of the spliced region; as a result, antisense probes specific for the spliced region could be synthesized using the polymerase site adjacent to the 3′ end of the insert. Following the synthesis reaction, the DNA template was removed by treatment with deoxyribonuclease I and the RNA probes recovered by two rounds of precipitation with ammonium acetate and ethanol. The FN-C, FN-EIIIA and FN-EIIIB probes were all then hydrolysed in alkali to a mean length of 150 nt (Cox *et al.* 1984), reprecipitated and finally dissolved in 50% formamide containing 10 mM-dithiothreitol (DDT). The FN-V probe was gel-purified from a denaturing polyacrylamide gel to remove the minor population of synthesized transcripts that included RNA complementary to the invariant region of FN included in the construct (generated by RNA synthesis from templates which the *Sau*3A restriction enzyme had failed to cut at the 5′ end of the V region) and then dissolved in the same solution.

In situ *hybridization*

The protocol used is a modification of those previously described by Cox *et al.* (1984), Hogan *et al.* (1986), Bandtlow *et al.* (1987) and Kintner & Melton (1987). Sections were dewaxed by placing the slides in xylene for 15 min and then rapidly rehydrated through an ethanol/water series. They were then placed in 0·2 M-HCl for 20 min, washed in 10 mM-Tris pH 7·6/1 mM-EDTA (TE) and treated with 1 μg ml^{-1} proteinase K in TE for 15 min at 37°C. The enzyme digestion was terminated with 0·2% glycine in phosphate-buffered saline (PBS) after which slides were washed briefly in PBS, fixed in 4% paraformaldehyde in PBS for 20 min, washed again in PBS and acetylated by placing them in 0·1 M-triethanolamine pH 8·0 containing 1/200 (v/v) acetic anhydride for 10 min. Slides were then placed in 2×SSC (1×SSC: 150 mM-NaCl, 15 mM-sodium citrate, pH 7·4) awaiting the addition of the hybridization mixture.

Hybridization buffer (50% deionized formamide, 10% dextran sulphate, 0·3 M-NaCl, 10 mM-Tris pH 7·6, 5 mM-EDTA, 0·02% (wt/v) Ficoll 400, 0·02% (wt/v) polyvinylpyrolidone, 0·02% (wt/v) bovine serum albumin, 10 mM-DTT, 100 μg ml^{-1} yeast tRNA and 500 μM-nonradiolabelled thio-UTP) was prepared immediately prior to use and the pH adjusted to 6·0 with HCl. The appropriate probe was added to a final concentration of 0·3 μg ml^{-1} per kilobase probe complexity, after which 50 μl of the mixture per slide was pipetted onto the sections. Coverslips, previously siliconized by dipping in 5% dimethyldichlorosilane in chloroform, were placed over the sections and the slides were incubated at 50°C within a humidified container for at least 15 h. Following this incubation, the coverslips were removed in two washes of 50% formamide, 2×SSC and 10 mM-DTT at 50°C. Slides were then transferred to TE/4×SSC and treated with ribonuclease (RNase A – Sigma type IIIA) at 10 μg ml^{-1} for 30 min at 37°C, after which they were washed again in TE/4×SSC, transferred into 50% formamide, 2×SSC and 10 mM-DTT for a stringent wash at 65°C for one hour and finally air-dried after passing through a graded series of alcohols containing 0·3 M-ammonium acetate.

For autoradiography, slides were dipped in Kodak NTB-2 emulsion which had been diluted 1:1 in water containing 2% glycerol and melted at 42–45°C. The slides were then dried and exposed for 7 days at −20°C, after which they were developed in Kodak D19 (1:1 in water) for 2·5 min, fixed in Kodak fixative for 5 min, washed, lightly stained with 0·02% toluidine blue, dehydrated and mounted with either Permount or DPX mountant. Slides were viewed with a Zeiss universal microscope equipped with bright-field and dark-field optics and photographs were taken using Tri-X film at 400 ASA or Tech Pan film at 85 ASA.

Results

(A) *FN synthesis by migrating cells*

In order to examine the distribution of FN mRNA during chick embryogenesis by *in situ* hybridization, we subcloned a 160-nucleotide cDNA sequence corresponding to a region of mRNA that is invariably included in the spliced transcript (Fig. 1) and inserted this shorter sequence into a pGEM plasmid. The construction of this plasmid, with SP6 and T7 polymerase sites at either end of the polylinker sequence, allowed the synthesis of antisense (to detect the FN mRNA) and sense (control) ^{35}S-labelled single-stranded RNA probes. Initial experiments established that the antisense probe (designated FN-C) hybridized specifically to sections of day-2 to -4 chick embryos and extraembryonic tissue (Fig. 2A,B), while no significant hybridization was seen with the sense probe (Fig. 2C).

Trunk neural crest cell migration

Trunk neural crest cells first appear in the dorsal neural tube as a population of neuroectodermal cells that lose attachment to their neighbours, leave the neural tube and migrate laterally between the neural tube and overlying ectoderm before following the different pathways throughout the embryo (see Le Douarin (1982) for review). When sections through the trunk region of stage-11 to -14 chick embryos showing initial neural crest cell emigration from the neural tube were hybridized with FN-C, heavy labelling was seen in the ectoderm overlying the neural

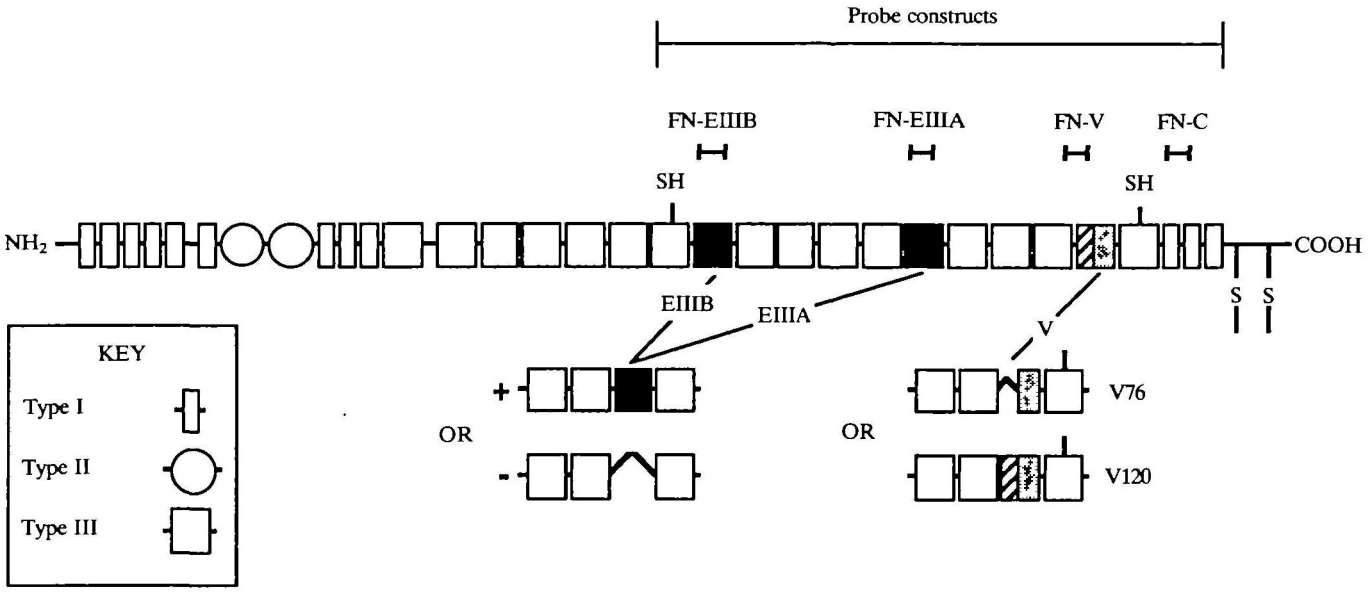

Fig. 1. A diagrammatic representation of the repeating modular structure of FN, showing the three regions of the protein altered by alternative splicing of the primary gene transcript, and illustrating the potential diversity of FN that alternative splicing could create. The regions corresponding to the cDNA sequences present in the probe constructs used in this study are marked; note that the mRNA sequence recognized by the FN-C probe is always included in the spliced transcript while the mRNA sequences recognized by the other three probes (FN-EIIIA, FN-EIIIB and FN-V) can each be alternatively spliced in or out of the final mRNA, yielding eight possible forms of chicken FN.

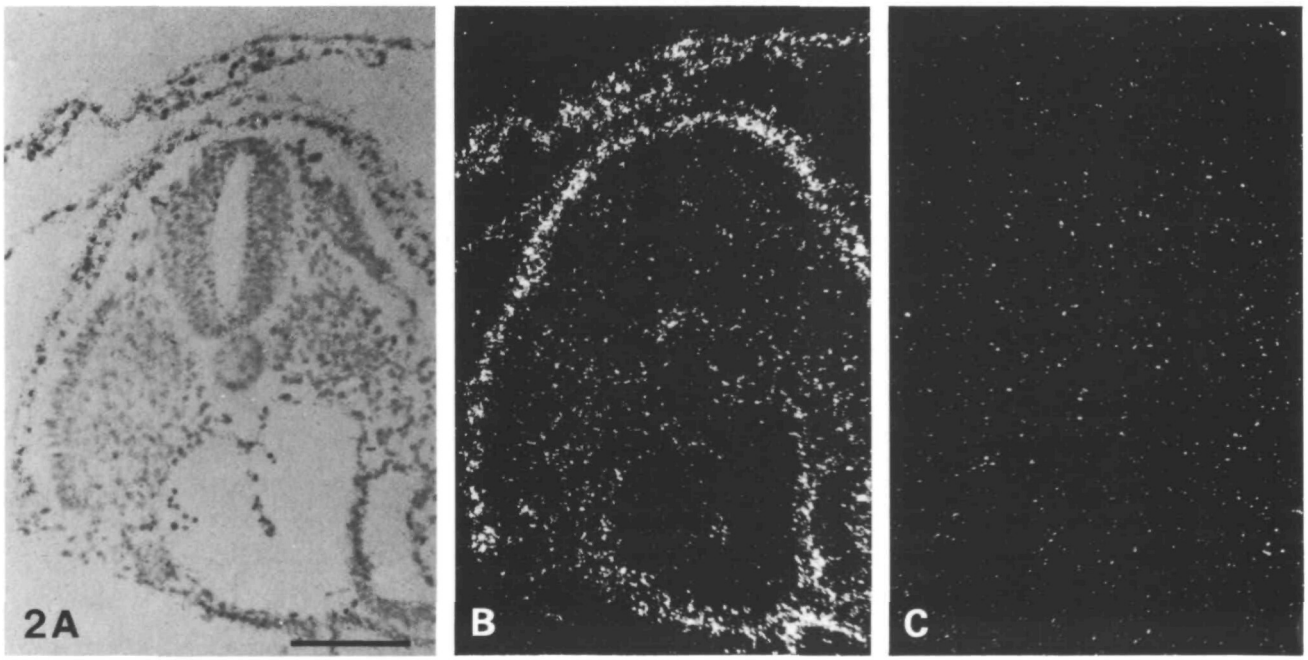

Fig. 2. Two adjacent transverse sections through the trunk region of a stage-16 chick embryo hybridized with antisense or sense FN-C probes and viewed with bright-field (A) and dark-field (B,C) optics. A and B show one section hybridized with the antisense probe while C shows the adjacent section hybridized to the sense probe. Note that specific hybridization to the embryo and overlying extraembryonic tissue is seen only with the antisense probe. Bar, 100 μm.

tube (Fig. 3). A much lower, but significant, level of labelling was also seen in the somites and neural tube, with no specific increase in labelling above this level associated with the dorsal part of the neural tube from which the neural crest cells originate (Fig. 3). Migrating neural crest cells themselves were unlabelled by the FN-C probe (Fig. 3). The absence of neural crest cell labelling did not reflect a nonspecific

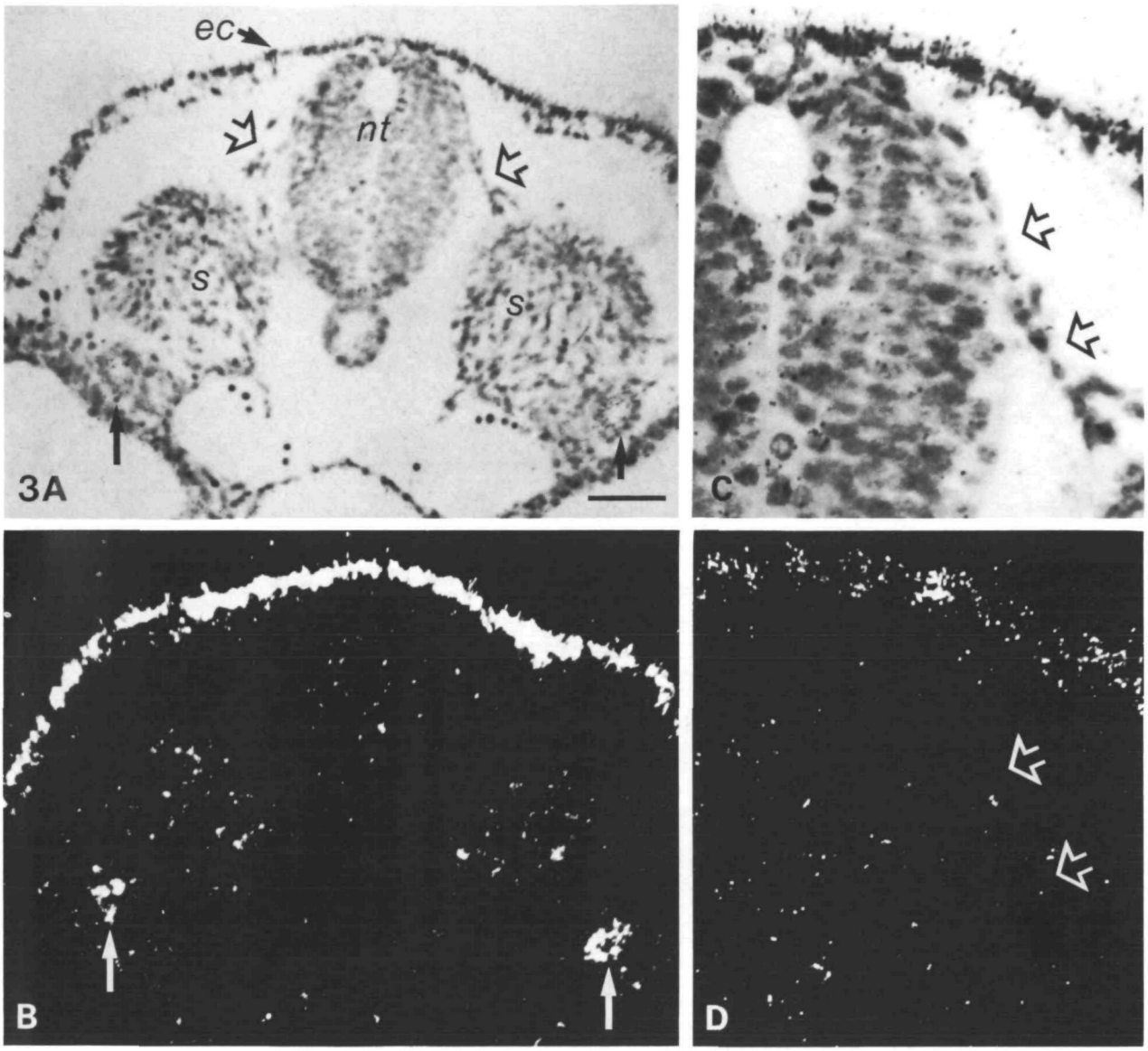

Fig. 3. A section through the trunk region of a stage-14 chick embryo hybridized with the FN-C antisense probe and viewed with bright-field (A,C) and dark-field (B,D) optics. The initial neural crest migration between the ectoderm (*ec*) and the dorsal surface of the neural tube (*nt*) can be seen in this section. Note that the ectoderm is heavily labelled and the neural tube and somites (*s*) are more lightly labelled. The neural crest cells (hollow arrows), in contrast, are unlabelled. This is more clearly shown in C and D, which show a magnified view of the neural crest cells (hollow arrows) on one side of the neural tube. The intensely labelled tubular structure near the ventral surface of the embryo is the mesonephros (arrows in A,B). Bar, 5 μm.

loss of mRNA from these cells during tissue processing, as both these cells and the neural tube were specifically labelled by a 800 nt probe complementary to the integrin β1 subunit mRNA (not shown) (Tamkun *et al.* 1986). Therefore migrating trunk neural crest cells appear not to synthesize FN, in keeping with previous *in vitro* studies (Newgreen & Thiery, 1980).

Area vasculosa mesenchymal cell migration

The extraembryonic vasculature of the stage-10 to -24 chick embryo consists of a network of vessels spreading over the surface of the yolk, with the outer limit of these vessels defined by a ring-shaped blood vessel (the terminal sinus) that migrates outwards between the two layers of yolk membrane, the epiblast (continuous with the embryonic ectoderm) and the yolk-rich endoderm. A small population of actively migrating mesenchymal cells precedes this blood vessel (Mayer & Packard, 1978; Flamme, 1987), migrating over an FN-rich basal lamina covering the inner surface of the epiblast (Fig. 4) (Mayer *et al.* 1981).

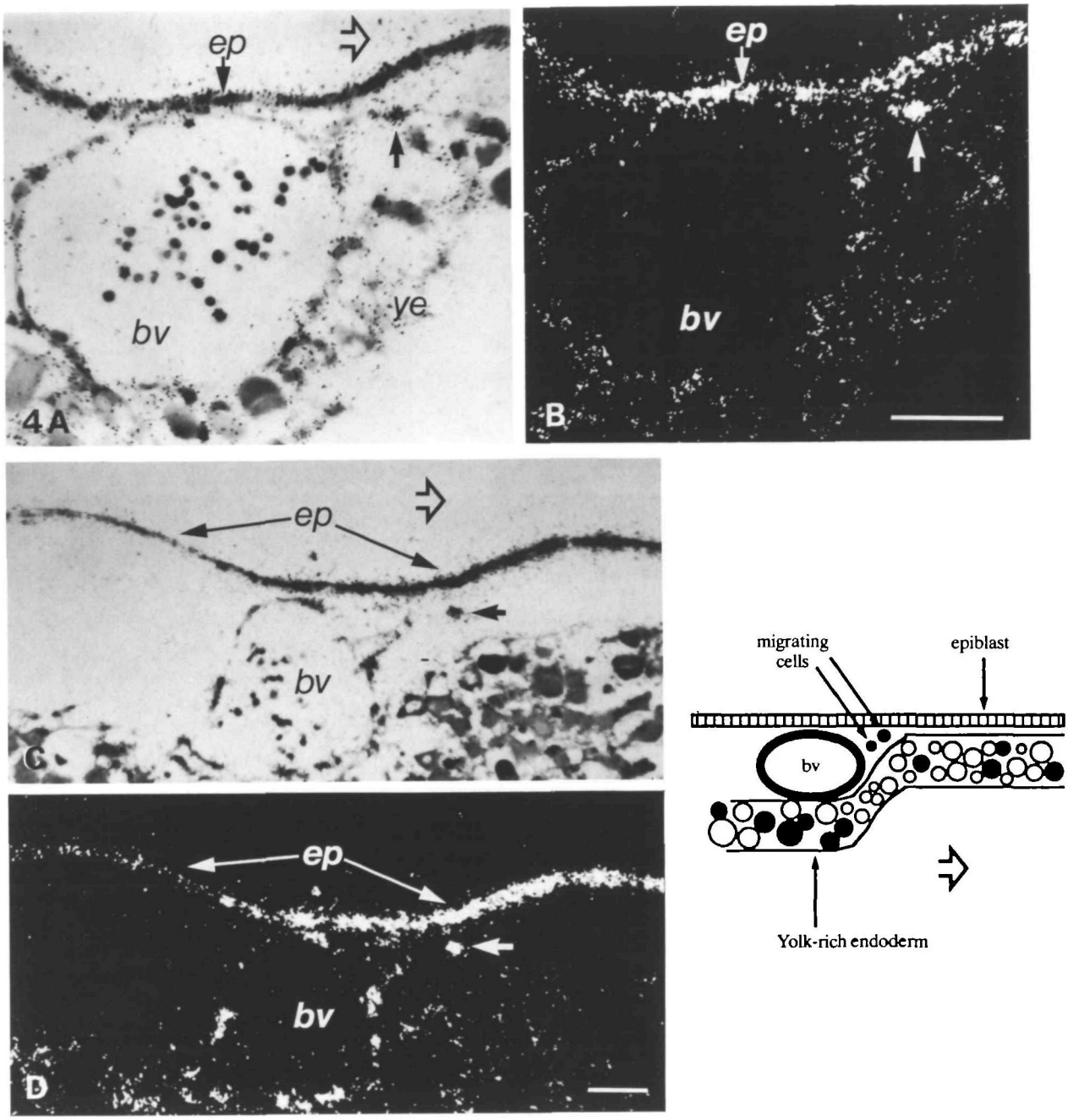

Fig. 4. Two sections through the area vasculosa region of a stage-14 chick embryo cut perpendicular to the blood vessel, hybridized with antisense FN-C probes and viewed with bright-field (A,C) and dark-field (B,D) optics. The anatomy of the region is shown schematically at the lower right corner of the figure, and can be seen in A and C and also in Fig. 9A. The migrating mesenchymal cells (solid arrow) precede the blood vessel (*bv*) moving away from the embryo between the epiblast (*ep*) and yolk-rich endoderm (*ye*). The hollow arrow indicates the direction of migration from left to right. Note that the antisense probe labels both epiblast and migrating cells intensely (A,B). Labelling is also seen over the vascular endothelium and, at a lower level, over the endoderm, but not over the cells within the blood vessel. C and D show a similar section viewed at lower power with bright-field (C) and dark-field (D) optics, illustrating the decrease in labelling of the epiblast after the migrating cells and blood vessel have moved over its surface. Bars, 50 μm.

Sections of this area taken from stage-16 to -24 chick embryos hybridized with the FN-C probe showed heavy labelling of the epiblast prior to mesenchymal cell arrival (Fig. 4A,B). Equally heavy labelling was seen over the migrating cells themselves and a lower level of labelling was observed over the endothelium

of the adjacent blood vessel and over the endoderm (Fig. 4A,B). The level of epiblast labelling diminished behind the migrating blood vessel (Fig. 4C,D), and remained at this lower level until the epiblast became the embryonic ectoderm, at which point labelling increased again (not shown). Therefore, in contrast with neural crest cells, the migratory cells of the area vasculosa appear to contain abundant FN mRNA.

Endocardial cushion cell migration

The heart initially develops as two tubes, one within the other, that first appear at stage 10–11 of development on the ventral surface of the embryo. The outer tube, connected to the embryo by the dorsal mesocardium, will form the myocardium while the inner tube becomes the endocardium. Initially, the space between the two tubes is cell-free and contains basal-lamina-like extracellular matrix material termed cardiac jelly (Kitten *et al.* 1987). Between stages 16–25 of development, endocardially derived cells at the atrioventricular junction and in the outflow tract migrate outwards across this space towards the myocardium (Markwald *et al.* 1977); these migrating cells form swellings, termed endocardial cushions, that function as primitive valves (Patten *et al.* 1948) and form the primordia of the adult heart valves (Van Mierop *et al.* 1963). Immunolocalization studies have demonstrated FN within the cardiac jelly prior to endocardial cushion cell migration and have shown a concentration gradient of FN-containing particles highest adjacent to the myocardium in those areas through which cell migration occurs (Kitten *et al.* 1987; Mjaatvedt *et al.* 1987).

Sections of stage-24 heart at the level of the outflow tract hybridized with the FN-C probe showed intense labelling of the endocardial cushion cells and of the endocardium from which they originated, but no labelling of the myocardium towards which they were migrating (Fig. 5). A similar pattern was seen in the atrioventricular endocardial cushion cell migration, although the level of labelling was lower in this region (not shown). When adjacent sections were hybridized with the probe complementary to the integrin $\beta 1$ mRNA, heavy labelling was seen of both endocardium and myocardium (not shown), confirming that the lack of myocardial labelling with the FN-C probe did not reflect a general loss of mRNA from this tissue layer.

In order to determine which cell types might be responsible for the FN present in the cardiac jelly prior to endocardial cushion cell migration, we examined the hybridization pattern of FN-C at a number of earlier stages of heart development. Soon after heart formation at stage 11, FN-C strongly labelled both myocardium and endocardium at the level of the outflow tract (Fig. 6A,B). More posteriorly, the level of myocardial labelling decreased slightly and was greatest on the dorsal regions of the myocardium adjacent to the dorsal mesocardium. Intense labelling was also seen in the floor of the developing foregut, which is in direct contact with the cardiac jelly at this stage (Fig. 6C,D). In subsequent stages, the level of myocardial labelling decreases uniformly, such that by stage 16 the myocardium at the level of the ventricle is barely labelled (Fig. 6E,F). Significant labelling remains at the level of the outflow tract at stage 16 (not shown), but has disappeared by stage 24

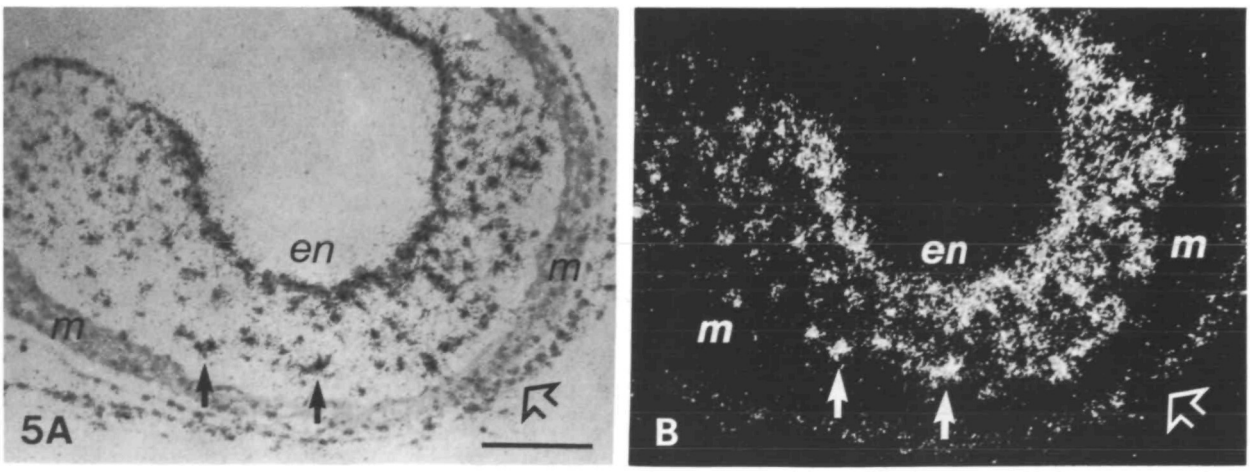

Fig. 5. A section through the outflow tract region of a stage-24 chick heart hybridized with the antisense FN-C probe and viewed with bright-field (A) and dark-field (B) optics. Many migrating endocardial cushion cells (two of which are arrowed) can be seen within the cardiac jelly between the endocardium (*en*) and myocardium (*m*). Note that the migrating cells and the endocardium are heavily labelled, while the myocardium is unlabelled. The weakly labelled layer outside the myocardium (hollow arrow) is the developing pericardium. Bar, 100 μm.

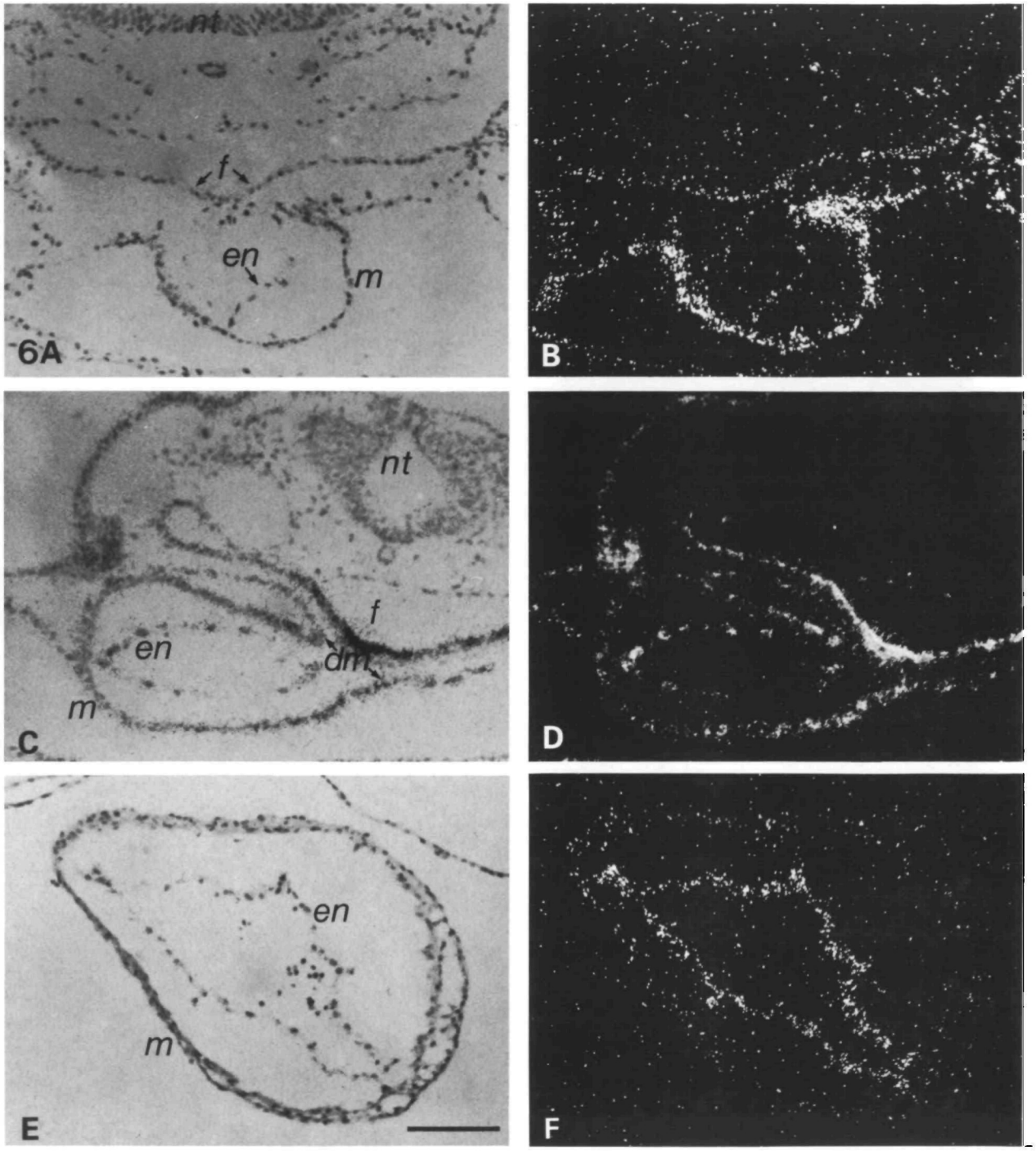

Fig. 6. Three transverse sections through the developing chick heart hybridized with the antisense FN-C probe and viewed with bright-field (A,C,E) and dark-field (B,D,F) optics. A and B is a section through the outflow tract region of a stage-11 heart, C and D is through the atrioventricular region of the same heart and E and F is through the ventricular region of a stage-16 heart. Myocardial (*m*) and endocardial (*en*) labelling can be seen in all these sections but at greatly differing levels, as explained in the text. *dm*, dorsal mesocardium; *f*, floor of the developing foregut; *nt*, neural tube. Bar, 100 μm.

(Fig. 5). The endocardium remains intensely labelled throughout all these stages (Figs 5 and 6). Thus, in the heart, the FN within the cardiac jelly could be synthesized by either the myocardium or the endocardium or both at stages prior to migration. During migration, however, the myocardial cells synthesize no FN while the migrating endocardial cushion cells contain abundant FN mRNA.

(B) *Alternative splicing of the FN gene transcript before and during migration*

The distribution of FN mRNA revealed by the FN-C probe suggests that in two different cell migrations, the mesenchymal cells of the area vasculosa and the endocardial cushion cells, the migrating cells may contribute additional FN to a matrix that already contains FN produced by cells adjacent to the migration pathway. In order to determine if the FN produced by the migrating cells differs from that produced by the cells of the pathway we next examined the pattern of alternative splicing of the FN gene transcript in these different cells.

We constructed plasmids containing short chicken cDNA sequences complementary only to the three alternatively spliced regions; EIIIA, EIIIB and the V region. Antisense RNA probes synthesized from these constructs were of lengths (230 nt, 160 nt and 144 nt, respectively) and uridine composition (23 %, 26 % and 27 %) similar to the 160 nt and 26 % uridine FN-C probe so as to allow comparisons to be made between the labelling levels on adjacent sections hybridized with the different probes at the same concentration. As with the FN-C probe, the antisense single-stranded RNA probes (designated FN-EIIIA, FN-EIIIB and FN-V) synthesized from these plasmids hybridized specifically to sections of stage-10 to -24 chick embryos while no specific hybridization was seen with sense probes from the EIIIA and EIIIB constructs. Sense probes could not be synthesized from the V region construct as explained in Materials and methods.

We verified that these probes could be used to detect patterns of alternative splicing by examining their hybridization to sections of embryonic day-17 liver, which has been shown previously to contain abundant FN mRNA coding for plasma FN from which EIIIA and EIIIB are almost entirely excluded, with the V region being excluded in about 50 % of the mRNA (Norton & Hynes, 1987). As expected, the FN-C probe hybridized strongly to the liver parenchyma, the FN-EIIIA and FN-EIIIB probes produced levels of parenchymal labelling only slightly greater than the sense controls, and the V region probe hybridized at an intermediate level (Fig. 7). These experiments also revealed a further cell-type-specific splicing difference within the liver. The walls of small vessels within the liver were intensely labelled by FN-EIIIA and FN-EIIIB, as well as FN-C, in marked contrast to the adjacent parenchyma (Fig. 7), showing the presence of mRNA coding for cellular FN (including EIIIA and EIIIB) in these embryonic vessel walls.

When these segment-specific probes were used to examine the patterns of FN gene splicing, widespread expression of all three segments was seen throughout the stage-10 to -24 embryo. Furthermore, no differences in the splicing of the FN gene transcript by the migrating cells as compared with the cells adjacent to their pathway could be detected in the developing heart between stages 10 and 24 or in the stage-16 area vasculosa. In all cases, the pattern and intensity of labelling in these cells appeared identical with that seen with the FN-C probe; for example, Fig. 8 shows the pattern of FN-V labelling of the endocardial cushion cells in the stage-24 outflow tract (compare with Fig. 5) while Fig. 9 shows the pattern of FN-EIIIB labelling during mesenchymal cell migration in the area vasculosa (compare with Fig. 4).

Discussion

The aim of this study was to elucidate the role of FN in the molecular mechanisms controlling cell migration by examining the patterns of synthesis *in vivo*.

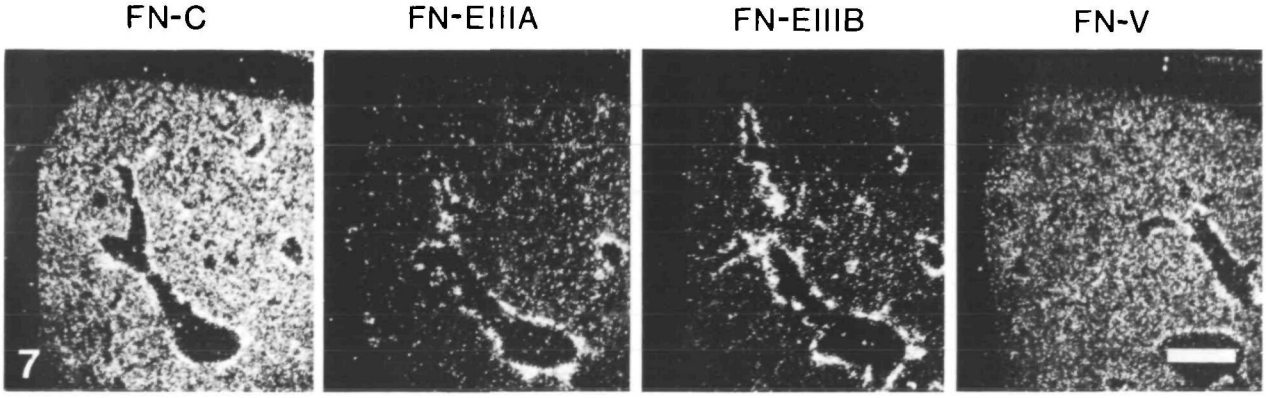

Fig. 7. Four closely adjacent sections of embryonic day-17 chicken liver hybridized with the antisense FN-C probe or the alternatively spliced segment-specific probes FN-EIIIA, FN-EIIIB or FN-V. All sections are viewed with dark-field optics and the probe used is shown above each photograph. Note that the parenchyma of the liver is strongly labelled only by FN-C and FN-V, while the walls of the vessels within the liver are intensely labelled by all the different probes. Bar, 200 μm.

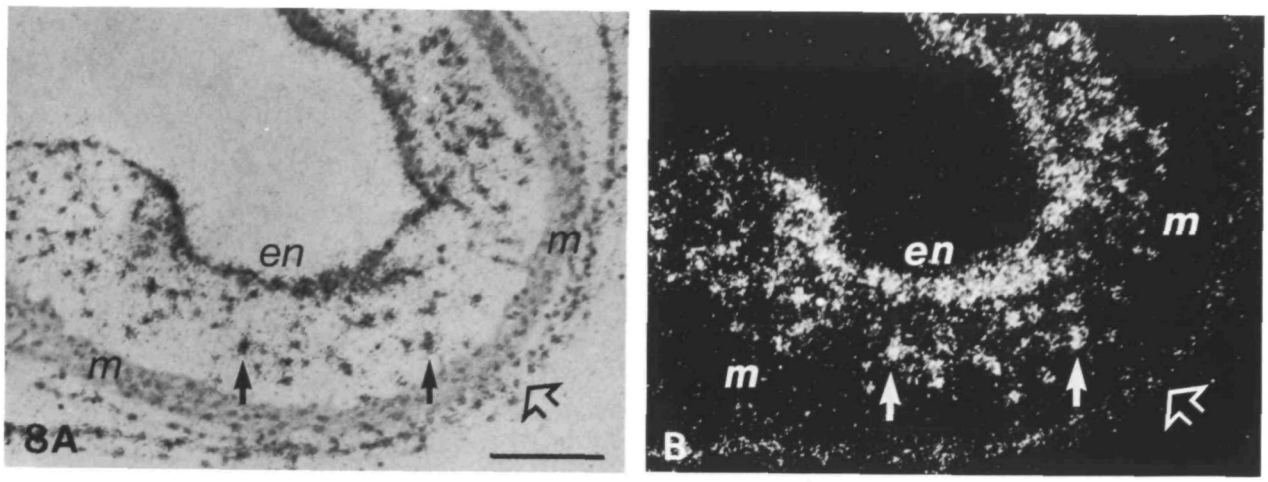

Fig. 8. A section of stage-24 heart adjacent to that shown in Fig. 5 hybridized with the antisense FN-V probe and viewed with bright-field (A) and dark-field (B) optics. Note that the pattern of hybridization is identical to that seen with the FN-C probe. Symbols as in Fig. 5. Bar, 100 μm.

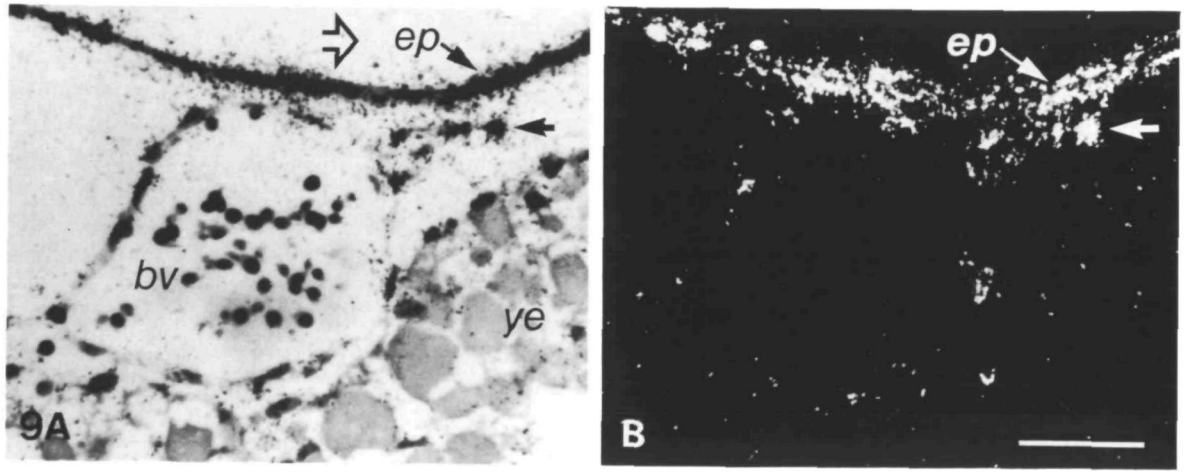

Fig. 9. A section of the area vasculosa region of a day-3 chick embryo similar to that shown in Fig. 4 hybridized with the antisense FN-EIIIB probe and viewed with bright-field (A) and dark-field (B) optics. Note that the migrating cells and the epiblast are intensely labelled, just as they were by the antisense FN-C probe. Symbols as in Fig. 4. Bar, 50 μm.

To do this we have used *in situ* hybridization to determine the distribution and patterns of alternative splicing of FN mRNA in three different cell migrations in the developing chick embryo; trunk neural crest cell migration, mesenchymal cell migration in the area vasculosa and endocardial cushion cell migration. The use of *in situ* hybridization offers two major advantages for the purposes of this study. First, in the case of a secreted, and possibly translocated, extracellular matrix molecule such as FN it provides unambiguous identification of the source(s) of the FN present in the migratory pathway, unlike previous *in vivo* immunolocalization studies. Second, it allows the construction of probes entirely specific to all the variably spliced regions of the FN gene transcript, including the EIIIB region which appears to be highly conserved between humans, rats and chickens (Schwarzbauer *et al.* 1987; Gutman & Kornblihtt, 1987; Norton & Hynes, 1987; Zardi *et al.* 1987) and to which we have so far been unable to generate an antibody. In interpreting these results, we assume that the presence of FN mRNA in these embryonic cells indicates that they synthesize and secrete FN. While formal proof of this assumption is lacking, a comparison of previous studies examining the relative amounts of the different alternatively spliced forms of FN synthesized by cultured hepatocytes (that largely make plasma FN) and by cultured fibroblasts and astrocytes (that largely make cellular FN) (Tamkun & Hynes, 1983; Price & Hynes, 1985; Paul *et al.* 1986) with studies examining the patterns of FN mRNA in these cells (Schwarzbauer *et al.* 1987) shows a very

good correlation between the levels of the different FN mRNAs and the synthesis of the corresponding FN proteins.

The first part of the study utilized a probe complementary to an invariant region of FN mRNA. We found that migrating trunk neural crest cells contain little, if any, FN mRNA immediately prior to, or during, their initial migration between the ectoderm and neural tube. The overlying ectoderm, by contrast, contains abundant FN mRNA, while a lower level is seen within the neural tube. The situation was quite different in the other two cell migrations that we examined; in the area vasculosa both the migrating cells and the epiblast cell layer express abundant FN mRNA and, in the developing heart, migrating endocardial cushion cells express high levels of FN mRNA as they migrate through the cardiac jelly, while the cell layers adjacent to this space also contain high levels of message earlier in development before the start of migration.

This apparent distribution of FN mRNA is largely consistent with previously reported immunolocalization studies of FN protein and extends these studies by providing evidence as to the sources of the observed FN *in vivo*. During neural crest cell migration, FN protein is detected by immunolabelling in the basement membranes of ectoderm, somites and neural tube, and in the adjacent matrix surrounding the migrating cells (Newgreen & Thiery, 1980; Thiery *et al.* 1982). *In vitro* studies show that cultured ectoderm, somite and neural tube synthesize FN while trunk neural crest cells in such experiments do not synthesize FN (Newgreen & Thiery, 1980). Our findings suggest that these *in vitro* studies accurately reflect the *in vivo* situation, and that trunk neural crest cells do not synthesize FN as they migrate.

Previous studies on mesenchymal cell migration in the area vasculosa have suggested that the migrating cells use the basement membrane of the epiblast as a migratory substrate (Mayer & Packard, 1978; Flamme, 1987), and that an FN-rich matrix is present on the inner surface of the epiblast over which movement of the migrating cells occurs (Mayer *et al.* 1981). These studies also suggested that FN is synthesized by the epiblast, as the FN appears on the epiblast surface before the arrival of the mesenchymal cells (Mayer *et al.* 1981). Our experiments support this interpretation, as the epiblast contains abundant FN mRNA before the passage of the migrating cells. However, our results in the area vasculosa reveal two unexpected findings. First, migrating mesenchymal cells themselves contain levels of FN mRNA similar to those seen in the epiblast, suggesting that they contribute FN to the matrix through which they migrate. Second, the level of FN mRNA in the epiblast diminishes immediately after the passage of the mesenchymal cells, raising the possibility that, once FN on the epiblast surface is no longer required for migration, the level of the mRNA within the cells is downregulated.

Endocardial cushion cell migration in the developing heart occurs through a cell-free space containing a basal-lamina-like extracellular matrix (Kitten *et al.* 1987). It has been suggested that FN provides a haptotactic gradient for cushion cell migration (Kitten *et al.* 1987) as FN-containing particles are present within the matrix in a concentration gradient highest adjacent to the myocardium (towards which the cells are migrating) (Kitten *et al.* 1987; Mjaatvedt *et al.* 1987). Our demonstration of FN mRNA in endocardium, myocardium and the floor of the developing foregut, as well as the migrating cushion cells themselves, is consistent with previous immunolocalization studies showing the presence of FN in all these areas (Waterman & Balian, 1980; Icardo & Manasek, 1983, 1984) and extends these studies by suggesting that all these tissues synthesize FN and might therefore contribute to the observed gradient of FN within the cardiac jelly. However, we observed that the level of FN mRNA in the myocardium decreased considerably between stages 11–16 (before the start of cushion cell migration) and was undetectable during cell migration at stage 24, while the endocardial level of FN mRNA remained uniformly high throughout these different stages. The seemingly paradoxical gradient of FN-containing particles (highest close to myocardium) prior to migration may reflect differences in the secretory behaviour of endocardium and myocardium: in studies examining the distribution of radiolabelled proteins within the cardiac jelly after culturing embryos in ^{3}H-amino acids, it was shown that the myocardium is a secretory tissue which contributes proteins to the cardiac jelly, while endocardial cell proteins remain localized to this cell layer (Krug *et al.* 1985). It seems possible, therefore, that the FN synthesized by the myocardium is secreted into the cardiac jelly to form the observed gradient, while that synthesized by the endocardium is incorporated only into the matrix adjacent to this cell layer. Alternatively, the gradient of FN-containing particles could arise from the trapping of secreted FN (from whatever source) by a gradient of some other molecule within the particle.

Perhaps the most striking result observed in this study is the presence of abundant FN mRNA in two different migrating cell types, the mesenchymal cells of the area vasculosa and the endocardial cushion cells. Given that both these cell migrations occur through areas already rich in FN, the question arises as to why they should also express FN mRNA, and therefore presumably synthesize FN and contribute it to the matrix. One possibility is that the FN present in

the matrix is different in some way from that made by the cell and that both these different forms are required for migration. In the second part of this study, we have approached this question using probes complementary to the variably spliced regions of the transcript and reexamined the FN mRNA in migrating area vasculosa mesenchymal cells and endocardial cushion cells to compare the splicing pattern with the FN mRNA within the cells adjacent to the migratory pathways. We were unable to detect any differences in these mRNAs, which appeared to include all three spliced regions in all cases, while we were able to detect differences in splicing in the developing liver. We therefore find no evidence that alternative splicing of the FN gene transcript is used as a mechanism to create differences between the FN secreted by migrating cells and by cells adjacent to the pathway, although we obviously cannot exclude that such differences arise either from a minor population of FN mRNAs undetectable by *in situ* hybridization or from post-translational modifications.

Our observation from these experiments with the segment-specific probes that all the variably spliced segments were widely expressed throughout stage-10 to -24 (day-2 to -4) embryos is consistent with the ribonuclease protection experiments reported by Norton & Hynes (1987), in which EIIIA$^+$ and EIIIB$^+$ forms of FN were found to predominate in embryonic extracts prepared between 2·5 and 8·5 days, and suggests an important role for these segments during the early stages of development. It will be of considerable interest to determine the composition of FN mRNA at later developmental stages so as to determine whether inclusion of any segments is temporally or spatially altered during development, and these studies are currently in progress.

Our demonstration that migrating trunk neural crest cells lack FN mRNA is in clear contrast with our findings in the area vasculosa and developing heart. The question as to why these migrating cell types show different patterns of FN synthesis has not been addressed in this study. It may reflect differences in the molecular composition of the various migratory substrates and/or differences in the role of FN in these cell migrations. Cell culture studies represent a promising approach to this question; for example, a recent study examining neural crest cells within three-dimensional gels of defined composition suggested that they neither synthesized nor required FN for migration (Bilozur & Hay, 1988), and it will be important to examine the requirements of other migratory cell types in such culture systems.

Returning to the question of molecular mechanisms controlling the *guidance* of cell migration, these *in situ* hybridization results suggest that the absence of FN synthesis by migrating trunk neural crest cells may indeed be a mechanism by which these cells increase their sensitivity to exogenous FN, as suggested by Newgreen & Thiery (1980). This would allow a guiding role for FN in the initial stages of trunk neural crest cell migration as the cells move between neural tube and ectoderm. However our results clearly show that such a simple mechanism cannot be operating in at least two other migratory systems, the mesenchymal cells of the area vasculosa and the endocardial cushion cells. These migrating cells contain abundant FN mRNA apparently identical to that present in adjacent pathway-forming cells, suggesting that they produce FN similar to that already present in the migratory pathway. These findings are consistent with an important role for FN in cell movement but not in cell guidance, as any such synthesis and secretion of FN might be expected to mask guidance cues provided by the exogenous FN.

This evidence for complexity in the control of cell migration is perhaps not surprising given the recognition of an increasing number of adhesive glycoproteins in addition to FN within the embryonic extracellular matrix. Moreover, these molecules may interact with FN to alter cell behaviour; it has recently been shown that the migratory behaviour of neural crest cells on FN can be inhibited by the addition of cytotactin (Tan *et al.* 1987), or chondroitin sulphate proteoglycan (CSPG) (Perris & Johansson, 1987) to the culture substrate, and that the behaviour of the cells can be manipulated by altering the ratio of cytotactin or CSPG to FN in the substrate. Whatever the role of FN alone, it therefore seems likely that other extracellular matrix molecules will also play a role in cell guidance and that the behaviour of the migrating cells will reflect the relative contribution of these different molecules to the extracellular matrix. Understanding the molecular mechanisms of cell guidance will therefore require a precise knowledge of the composition of the pathway matrix, the relative contributions made to this matrix by migrating and pathway-forming cells and the patterns of expression of cell-surface receptors for these extracellular matrix molecules on the migrating cells themselves.

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