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Spatiotemporal patterns of fibronectin distribution during embryonic development I. Chick limbs

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SUMMARY

It has been suggested that an extracellular matrix – and cell surface – associated glycoprotein, fibronectin, plays a role in the positioning of cells in morphogenesis and in the maintenance of orderly tissue organization. In the present study the appearance and distribution of fibronectin during *in ovo* chick limb development has been investigated by indirect immunofluorescence techniques in H.H. stages 20–30. Fibronectin is not detectable until just prior to the transition from the morphogenetic to the cytodifferentiation phase of development. Beginning at H.H. stage 25, successive nonrandom patterns of fibronectin detection and distribution, which resemble the subsequent cartilaginous elements, precede overt chondrogenesis as detected by Alcian blue staining. This corresponds to the onset of the cytodifferentiation phase of limb development. As the accumulation of acidic proteoglycan increases in the cartilage matrix and the mesenchymal cells become more round in appearance, the presence of detectable fibronectin decreases and is ultimately seen only in the perichondria and basement membrane. However, predigestion of developed cartilage tissue with testicular hyaluronidase, prior to fibronectin staining, indicated that fibronectin remains a major constituent of cartilage matrix and is apparently masked by cartilage-specific proteoglycans. This study of chick limb development is consistent with the hypothesis that fibronectin may be a molecule that facilitates the spatial organization of cartilaginous primordia cytodifferentiation.

INTRODUCTION

The chick limb bud forms as a small elevation from the body wall and initially contains a primitive mesenchyme covered by ectoderm. Prior to Hamburger-Hamilton stage 25 is referred to as the morphogenetic stage of development (Zwilling, 1968). The first cytologic change that identifies mesenchymal cells differentiating in chondrogenesis is cell condensation resulting in the formation of a central blastema which resembles a miniature model of the future limb

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skeleton (Thorogood & Hinchliffe, 1975). Cartilage develops from the condensed mesenchyme (Gay & Miller, 1978). Around stage 25 there is a gradual transition from the morphogenetic stage to the cytodifferentiation stage of development. This is characterized by increased activity of hyaluronidase, degradation of hyaluronate, increased synthesis of chondroitin sulfate, increased synthesis of Type II collagen, and metachromasia of the cartilage extracellular matrix (Searls, 1965; Toole, 1972; Linsenmayer, Toole & Trelstad, 1973; Dessau, Von Der Mark, Von Der Mark & Fischer, 1980). The perichondrium forms the outer limit of the cartilaginous skeleton. The entire proximodistal sequence of the laying down of the cartilaginous skeleton primordia occurs between days 4 and 7 of incubation or stages 24 through 30 (Thorogood & Hinchliffe, 1975).

During early mesenchymal cell proliferation and development these cells form a syncytial network with fibrillar elements between the cells that may be characterized as an initial extracellular matrix (Gay & Miller, 1978). A major component of the fibrillar matrix, fibronectin, has been identified in the loose connective and primitive mesenchymal tissue of developing chick embryos *in ovo* (Linder, Vaheri, Ruoslahti & Wartiovaara, 1975), as well as in the extracellular matrices secreted by chick embryo fibroblasts *in vitro* (Chen *et al.* 1978). The extracellular matrix – and cell surface – associated glycoprotein fibronectin has been purported to be a 'differentiation antigen' because the appearance of fibronectin was found to be a property of primitive mesenchymal cells acquired early in ontogenesis, but was not visualized during subsequent differentiation (Linder *et al.* 1975).

Although there is no direct evidence for the function of fibronectin during *in vivo* development, it may play important roles in cell adhesion (Pearlstein, 1976; Yamada, Olden & Pastan, 1978), maintenance of cellular morphology (Yamada *et al.* 1978), and induction of cell migration (Ali & Hynes, 1978), as well as cellular differentiation (Zetter, Martin, Birdwell & Gospodarowicz 1978). During chondrogenesis *in vitro*, for example, the mesenchymal cells lose their close cell contacts, and with the development of a rounded chondrocyte morphology, fibronectin is no longer accumulated (Lewis, Pratt, Pennypacker & Hassell, 1978). Conversely, culturing chondroblasts in the presence of high levels of fibronectin causes them to assume a fibroblastic morphology, with a loss of synthesis of α_2 collagen chains typical of fibroblastic or early limb-bud cells (West *et al.* 1979; Pennypacker, Hassell, Yamada & Pratt, 1979). Thus, some investigators have concluded that the appearance of fibronectin in fibroblasts is correlated with and is a phenotypic marker of cytodifferentiation (Wartiovaara, Stenman & Vaheri, 1976), and may even be related to cell patterning during organogenesis (West *et al.* 1979; Newman & Frisch, 1979).

Preliminary studies in our laboratory of embryonic chick limb development (Melnick *et al.* 1980) were consistent with previous suggestions that fibronectin may have a role in the spatial organization of mesenchymal cytodifferentiation (West *et al.* 1979; Newman & Frisch, 1979). Our initial observations prompted

us to extend our studies in an attempt to further understand the association between fibronectin and *in ovo* mesenchymal cytodifferentiation.

In the present study the appearance and distribution of fibronectin during chick limb development *in ovo* has been investigated by indirect immunofluorescence techniques in H.H. stages 20–30. Successive nonrandom patterns of fibronectin detection and distribution, which resemble the subsequent cartilaginous elements, precede overt chondrogenesis as detected by Alcian blue staining. Pretreatment of developed cartilage tissue with testicular hyaluronidase, prior to fibronectin staining, indicated that fibronectin remains a major constituent of cartilage matrix and is apparently masked by cartilage proteoglycans.

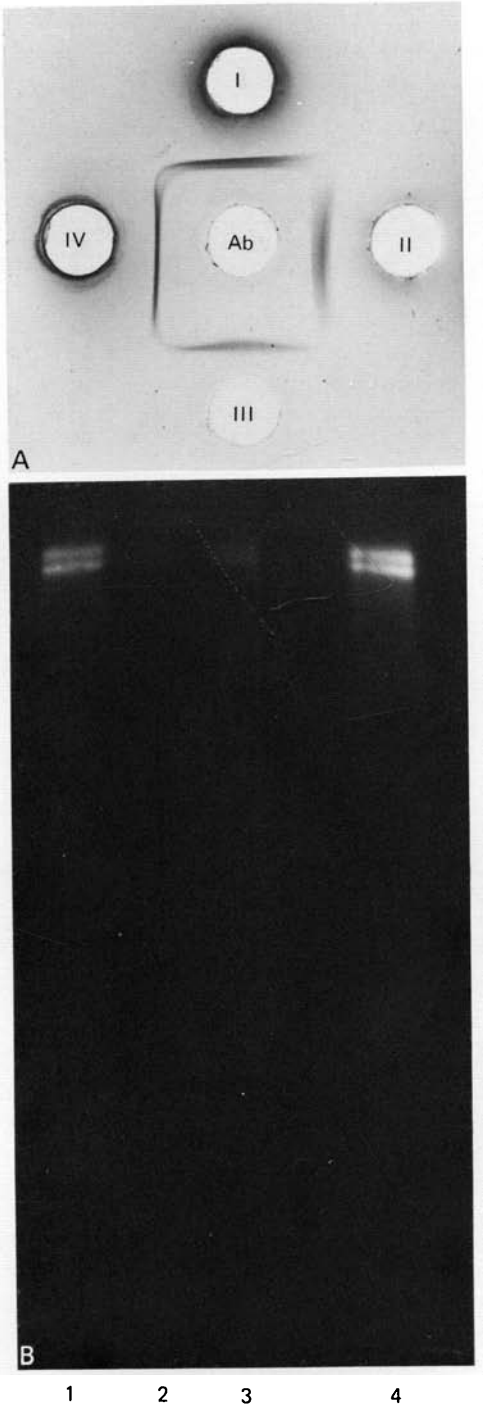
MATERIALS AND METHODS

Histologic procedures

White Leghorn chick embryos were incubated at 37·5 °C for various times to provide H.H. (Hamburger & Hamilton, 1951) stages 20–30 at the time of harvesting. Hindlimbs were removed with microscalpels to include part of the body wall, washed twice in phosphate-buffered saline (PBS), fixed in 4 % formalin (4 °C for 24 h), and transferred to 30 % sucrose (4 °C for 24 h). Fixed hindlimbs were embedded in Tissue-Tec (Miles Laboratories, Inc., Naperville, IL) at −20 °C and serially sectioned dorsoventrally in a cryostat at 4 μm along the proximodistal axis to allow visualization of fibronectin distribution during the entire proximodistal sequence of limb development. Sections were placed on clean albumin-glycerin coated slides and post-fixed 5 min in 95 % ethanol. Sections were incubated with a 1:5 dilution of rabbit anti-mouse fibronectin antibody (5·8 μg/50 μl/section) for 30 min at room temperature, washed three times (10 min each) with PBS (pH 7·1), incubated with a 1:20 dilution of FITC-labelled goat anti-rabbit 7S globulin (Hyland, Deerfield, IL) for 30 min at room temperature, and then washed three times (10 min each) with PBS (pH 7·1). The following controls were routinely utilized at all H.H. stages: (1) testing of fluorescein conjugate to ensure that no nonspecific fluorescence was involved; (2) use of preimmune serum in place of antibody. The mounted sections were examined with a Zeiss Epifluorescence photomicroscope equipped with a high-pressure mercury burner (HBO-50) and a tungsten lamp (12 V, 60 W). Controls were routinely negative for fluorescence.

For purposes of technical comparison, embryos at selected stages were fixed overnight in absolute alcohol–glacial acetic acid (99:1) (4 °C), dehydrated in absolute alcohol (4 °C for 1 h), cleared in xylene (4 °C for 1 h), and embedded in paraplast (59 °C). Tissue was serially sectioned at 7 μm in a plane identical to that used with the cryostat sections. Sections were placed on clean albumin-glycerin coated slides and stored at −20 °C. After deparaffinization, the procedure for fibronectin staining was the same as that used for cryostat sections.

In consecutive serial sections, the binding of Alcian blue at low pH (1·8) by


I
IV
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the cartilage matrix was used to assess the extent of chondrogenesis in the specimens through H.H. stage 30 (Lev & Spicer, 1964). Mounted sections were examined and photographed with a standard Zeiss photomicroscope. Finally it should be noted that selected serial sections at H.H. stage 30 were incubated with a sodium acetate buffered (pH 5·0) solution (1 mg/50/μl/section) of ovine testicular hyaluronidase type III (Sigma Chemical Co., St Louis, Mo) prior to fibronectin staining in order to determine if fibronectin was either truly absent from cartilage or merely masked by proteoglycans such as chondroitin sulphate.

Antibody preparation and specificity

Fibronectin was prepared from pooled citrated mouse plasma on an affinity column of gelatin-Sepharose 4B (Engvall & Ruoslahti, 1977). Antiserum was raised in rabbits and purified antibodies obtained by affinity chromatography using fibronectin-Sepharose 4B (Ruoslahti, Vuento & Engvall, 1978). Specificity of the antibodies was demonstrated by double diffusion in Agarose gel (Ouchterlony, 1958) (Fig. 1A) and by radioiodination of the fibronectin with the Bolton-Hunter reagent (Bolton & Hunter, 1973), reaction of the antibodies with the radiolabelled protein, and precipitation of the complex with goat anti-rabbit 7S γ-globulin (Hyland, Deerfield, Illinois) followed by SDS polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was done using the method of Ehrlich (1979) with the slight modification that the Tris buffer was adjusted to pH 8·9. The gel was not stained but immediately washed with 5% trichloroacetic acid to fix the proteins, dried, and exposed to X-omat R film (Kodak) for 2 days (Fig. 1B).

Fig. 1. (A) Ouchterlony analysis of antibody to mouse plasma fibronectin. Purified antibody (40 μl) was placed in central well (Ab). Two different samples of mouse plasma (40 μl each) were placed in wells numbered 1 and 4. The isolated immunogen (40 μl) was placed in wells numbered 2 and 3. After overnight incubation at room temperature the gel was exhaustively washed with borate buffered saline to remove unprecipitated materials. The gel was stained with 1% acetic acid then photographed using transmitted light.

(B) SDS-polyacrylamide slab gel electrophoresis (6%) and autoradiography of (lane 1) ^{125}I-labelled original immunogen; (lane 2) immunoprecipitate using only goat anti-rabbit IgG (= secondary antibody); (lane 3) supernatant of reaction mixture between secondary antibody and original immunogen and (lane 4) precipitate obtained when antibody to fibronectin was reacted with ^{125}I-fibronectin and this reaction product precipitated with goat anti-rabbit IgG. The supernatant from this reaction showed no bands on the autoradiogram and is not included. The relatively less intense bands seen in lane 3 are due to the fact that the three washings of the immunoprecipitate were not added to the supernatant fraction prior to lyophilization, resolubilization and electrophoresis. The area between lanes 3 and 4 was left empty to serve as a spacer between the two reaction mixtures.

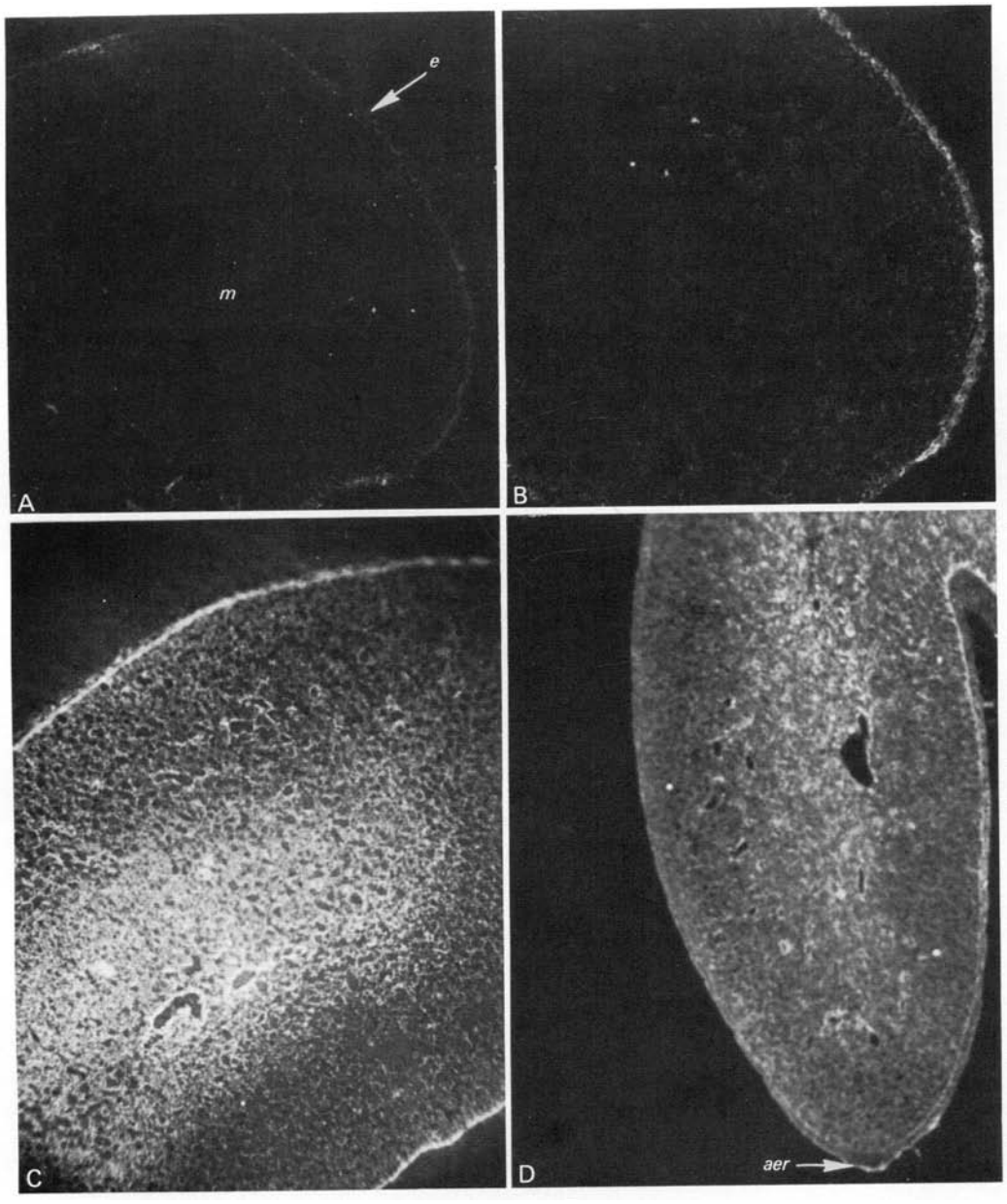

Fig. 2. Immunofluorescent localization of fibronectin during chick limb-bud development, H.H. stages 20–25. Figures A–C are oriented proximal to the left, distal to the right; figure D is oriented proximal to the top, distal to the bottom. (A) H.H. stage 20 (×72); (B) H.H. stage 22 (×72); (C) H.H. stage 25 (×89); (D) H.H. stage 25 (×80) – the fibronectin staining pattern of this paraffin-embedded limb bud is identical to that observed with frozen sections (compare with 2C). Key: *e*, ectoderm; *m*, mesenchyme; *aer*, apical ectodermal ridge. See text for full explanation of figures.

RESULTS

Early limb-bud development

The appearance and distribution of fibronectin in developing limbs from H.H. stages 20–25 was investigated. H.H. stages 20–21 (Fig. 2A) do not contain detectable mesenchyme fibronectin. At H.H. stage 22 (Fig. 2B) there is detectable fibronectin which appeared as diffuse, but minimal, staining in the area subjacent to the ectoderm; at stage 23 the staining is intensified and the diffuse pattern begins to centralize. By H.H. stage 25 (Fig. 2C, D) the staining pattern is well defined and resembles the future cartilagenous primordium of the femur. To this point Alcian blue staining is negative.

Late limb-bud development

At stage 26 the well-defined staining pattern for fibronectin resembles the future cartilagenous primordia of the tibia and fibula (Fig. 3A). Also at this stage there is the first minimal detection of cartilage matrix by Alcian blue (Fig. 3B) staining. By stage 29 (Fig. 3C) the tibia, fibula, and fibulare are defined cartilagenous primordia with the perichondrium stained by fibronectin antibody. The fourth distal tarsal, proximal tibial centrale, and third and fifth metatarsals are represented by discrete areas of intense fibronectin staining. Alcian-blue staining is negative to weakly positive in these areas. The fourth metatarsal represents a state of differentiation midway between the tibia and proximal tibial centrale.

Figure 4 depicts development in H.H. stage 30. By stage 30 Alcian blue staining (Fig. 4A) of cartilage matrix can be detected to varying degrees in the tibia, fibula, fibulare, proximal and distal tibial centrale, distal tarsals, metatarsals 2, 3, 4, and phalanges 2, 3, 4. Fibronectin staining (Fig. 4B, C, D), other than the perichondria, is absent from all structures except the tibial centrale, fifth metatarsal, and phalanges 2, 3, 4. The cartilage primordia of these structures will become more evident by 8–9 days of development (Romanoff, 1960). Hyaluronidase pretreatment of analogous sections of stage-30 limb buds prior to fibronectin staining indicated that fibronectin was still present in more developed cartilage matrix (Fig. 5) and that the absence of fibronectin staining in non-pretreated cartilage was probably the result of masking by cartilage proteoglycans. Treatment of stage-30 hindlimbs with boiled (inactivated) enzyme did not enhance the fibronectin staining in mature cartilage matrix.

DISCUSSION

Among the most intractable problems that have occupied the thoughts and time of experimental embryologists are those involving embryonic pattern formation. Pattern formation is concerned with the spatial organization of

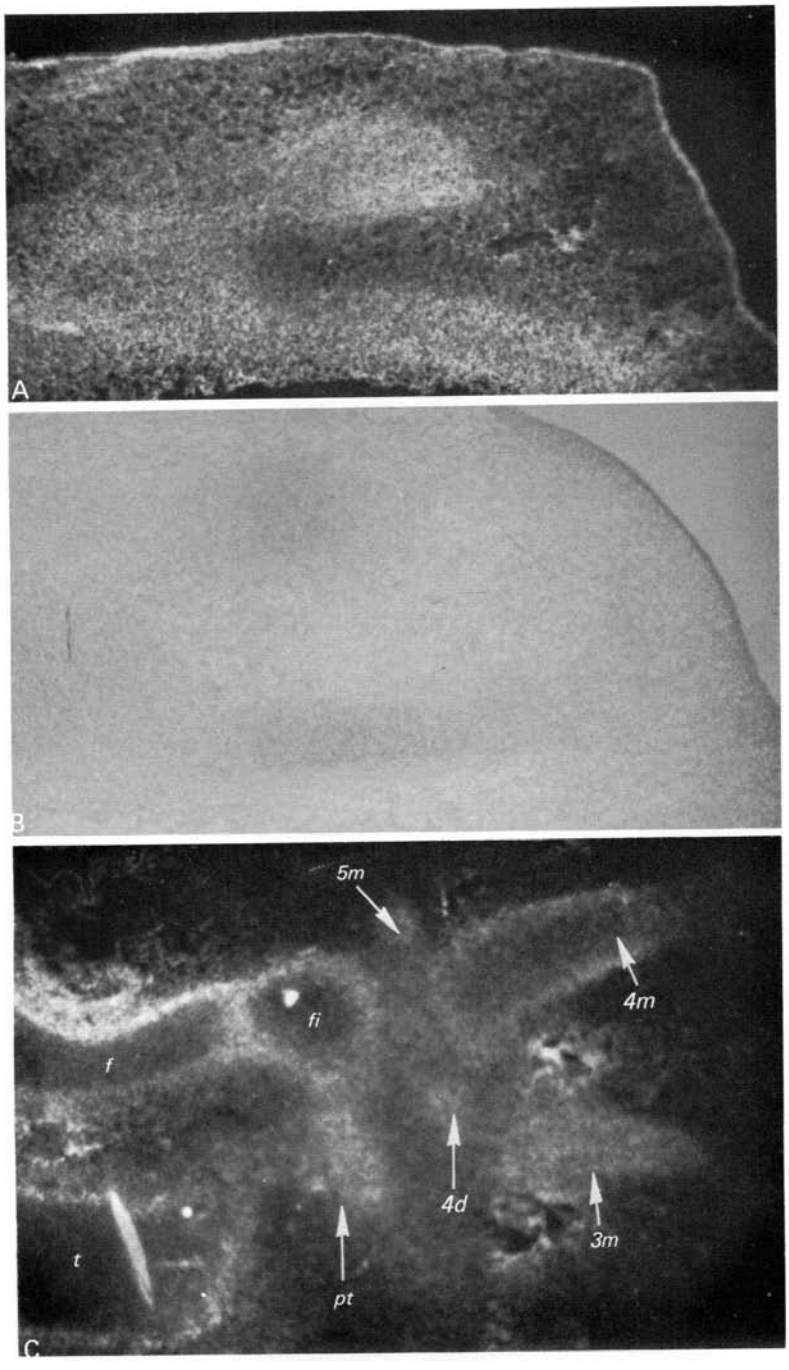

Fig. 3. Immunofluorescent localization of fibronectin during chick limb-bud development, H.H. stages 26–29. All figures are oriented proximal to the left, distal to the right. (A) H.H. stage 26 (× 56); (B) H.H. stage 26 (× 79); (C) H.H. stage 29 (× 64). Key: *f*, fibula; *fi*, fibulare; *pt*, proximal tibial centrale; *t*, tibia; *4d*, fourth distal tarsal; *3m*, third metatarsal; *4m*, fourth metatarsal; *5m*, fifth metatarsal. See text for full explanation of figures.

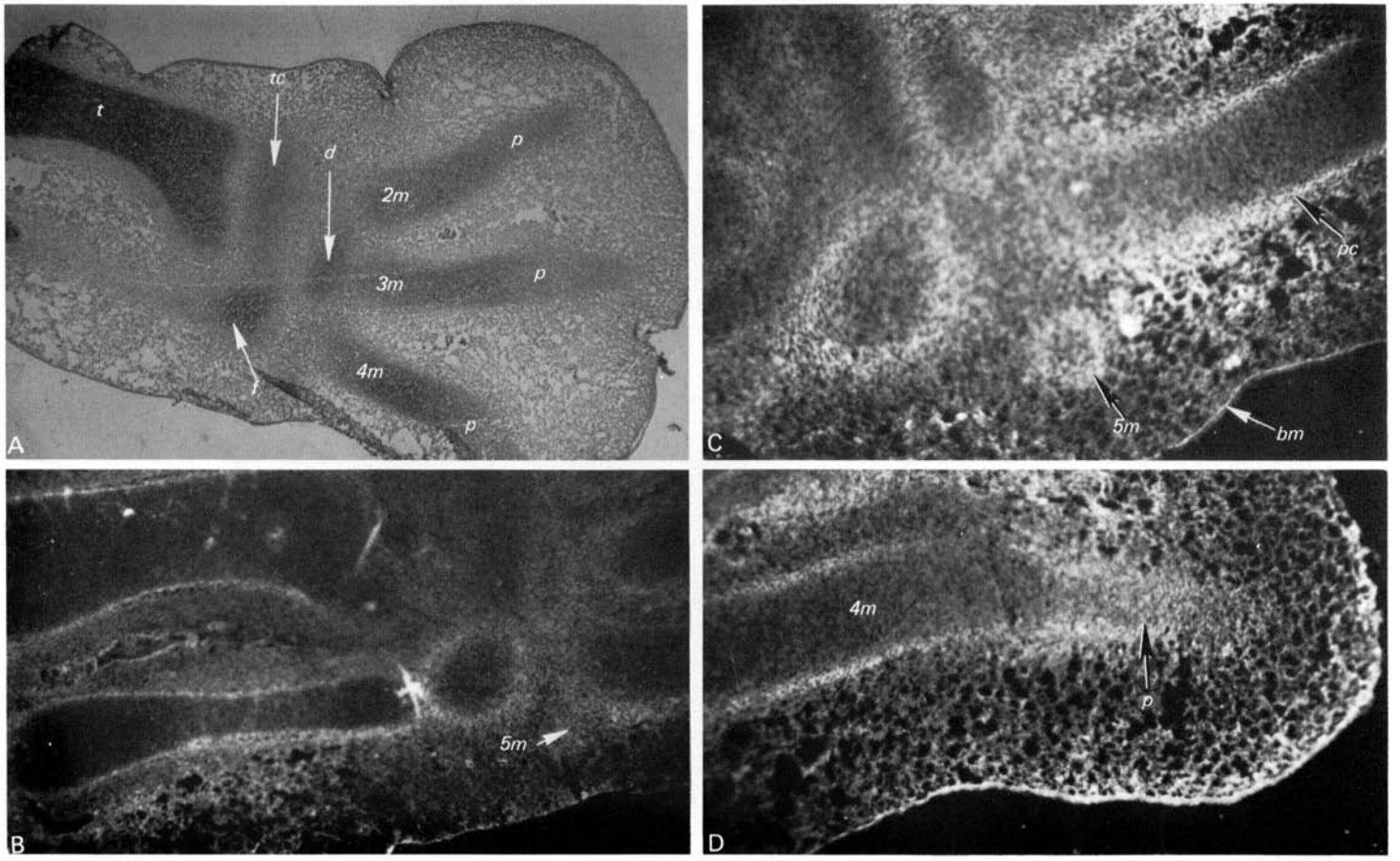

Fig. 4. Chick limb development, H.H. stage 30. (A) Alcian blue staining (×39); (B–D) fibronectin staining. All figures oriented proximal to the left, distal to the right (B) ×54; (C) ×90; (D) ×87 Key: *t*, tibia; *f*, fibula; *tc*, proximal and distal tibial centrale; *d*, distal tarsals; *2m*, second metatarsal; *3m*, third metatarsal; *4m*, fourth metatarsal; *5m*, fifth metatarsal; *p*, phalanges; *pc*, perichondrium; *bm*, basement membrane See text for full explanation of figures

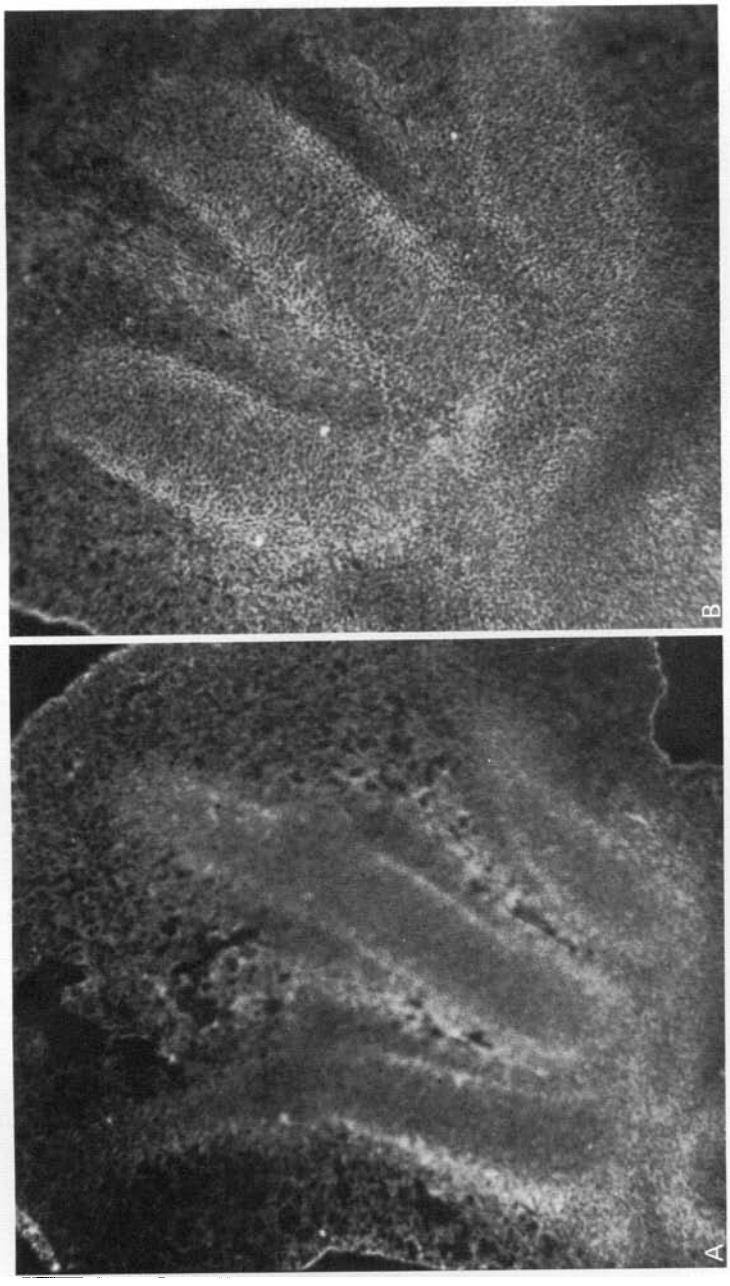

Fig. 5. Hyaluronidase pre-treatment of similar sections of H.H. stage-30 developing digit region. (A) Non-pre-treated control section (×53); (B) hyaluronidase pre-treated section demonstrating the presence of fibronectin in cartilage matrix (×95). See text for full explanation of figures.

cytodifferentiation (Wolpert, 1978). The concept of positional information has been applied extensively to pattern formation over the last decade (Summerbell, Lewis & Wolpert, 1973; Wolpert, 1978), particularly to limb development. The premise is that pattern formation may result from cells first having their positions specified with respect to boundary regions (e.g. a coordinate system) after which the cells interpret this positional information according to their genome and developmental history (Wolpert, 1978). Although the molecular basis of positional information and the interactions involved in the pattern formation are largely unknown, it has been suggested that fibronectin plays a role in the positioning of cells during morphogenesis and in the maintenance of orderly tissue organization (Wartiovaara, Leivo, Virtanen & Vaheri, 1978).

Recently, West *et al.* (1979) presented a model for pattern formation in chick limbs in which fibronectin plays a major role. They have found that fibronectin reversibly blocks the expression of the chondroblastic phenotype and induces a fibroblastic phenotype in chondroblasts *in vitro*. They noted that fibronectin does not interfere with myogenic differentiation *in vitro* (Chen *et al.* 1978), a process which takes place in a different region of the chick limb bud *in ovo*. West *et al.* (1979) concluded that these observations were consistent with the possibility that fibronectin is a negative control for chondrogenesis in nonchondrogenic regions of the limb bud. They also noted that their model requires, *a priori*, the disappearance of fibronectin from chondrogenic regions.

Newman & Frisch (1979) have presented an alternative model for the generation of the proximodistal sequence of skeletal elements during the development of the chick limb. They proposed that the biosynthesis and nonrandom diffusion through the extracellular matrix of a cell surface protein (e.g. fibronectin) will lead to specific spatial patterns of this molecule that could be the basis of spatial organization of cytodifferentiation and thus the emergent cartilagenous primordia. Their model predicts that as cellular differentiation proceeds, there will be a sequential reorganization of the 'morphogen' (e.g. fibronectin) pattern. Furthermore, the successive patterns of 'morphogen' distribution in competent tissue would precede overt chondrogenesis. In contrast to the model of West *et al.* (1979) fibronectin would be a positive control for chondrogenesis.

Data exists which can be viewed as supporting one or the other of the models discussed above. The recent study by Dessau *et al.* (1980) would seem to at least partially support the model of West *et al.* (1979). Dessau *et al.* (1980) found that fibronectin was evenly distributed throughout the intercellular space of the mesenchyme prior to condensation of core mesenchyme of the limb anlage and formation of the cartilage blastema. As cartilage differentiation progressed, fibronectin disappears and is completely absent from mature cartilage. In this regard, the results of the study by Dessau *et al.* (1980) appear to contrast sharply with those presented in this study. However, their study

is neither strictly comparable to that presented here nor does it really speak to the models proposed for two reasons: (1) the immunohistology does not allow for ready visualization of fibronectin distribution during the entire proximodistal sequence of limb development; (2) stages of limb development in which mature cartilage was present were not pretreated with testicular hyaluronidase in order to determine whether the fibronectin is really 'absent' or merely masked by the presence of cartilage-specific proteoglycans.

Our studies of chick limb development are more consistent with the hypothesis of Newman & Frisch (1979) that fibronectin may be a molecule that facilitates the spatial organization of cartilaginous primordia cytodifferentiation. Both our studies and those of Dessau *et al.* (1980) show that fibronectin can be detected prior to the earliest appearance of cartilage-specific Type-II collagen at late stage 24 (Dessau *et al.* 1980). In contrast to Dessau *et al.* (1980), however, our studies indicate that fibronectin is not detectable until just prior (stage 22, not stage 19) to the transition from the morphogenetic to the cytodifferentiation phase of limb development, and then it is distributed in a specific, non-uniform manner in the limb-bud mesenchyme. Beginning at H.H. stage 25, fibronectin accumulates in a pattern which closely resembles the subsequent cartilaginous element. This corresponds to the onset of the cytodifferentiation phase of limb development (Toole, 1972; Linsenmayer *et al.*, 1973). Successive non-random patterns of detectable fibronectin distribution reach maximum density just prior to overt chondrogenesis. As the accumulation of acidic proteoglycan increases in the cartilage matrix and the mesenchymal cells become more round in appearance, the presence of detectable fibronectin decreases and is ultimately seen only in the perichondria and basement membrane. However, predigestion with testicular hyaluronidase reveals that fibronectin is still present in cartilage, probably masked by proteoglycans. A similar masking of fibronectin in developing cartilage and bone has been found when *de novo* bone formation was induced with demineralized bone matrix (Weiss & Reddi, 1980). In summary, the present study is consistent with the idea that fibronectin is a positive control of developing cartilaginous structures.

Although our results suggest a positive association between fibronectin and pattern formation in the developing chick limb, the evidence is circumstantial, not direct. Furthermore, a number of other macromolecules are very likely to be associated with pattern formation. For example, the increased synthesis of hyaluronate during the morphogenetic phase has led to the hypothesis that hyaluronate is associated with the migration and proliferation of mesenchymal cells and with inhibition of their differentiation (Toole, 1972). Finally, the function of fibronectin during development is not known. Studies of early chick development *in ovo* suggest that the function of fibronectin is to facilitate morphogenetic movement during tissue organization (Critchley, England, Wakely & Hynes, 1979), but direct evidence remains to be found.

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