Int. J. Dev. Biol. 37: 369-380 (1993)

Original Article

Localization of RIHB (retinoic acid-induced heparin-binding factor) transcript and protein during early chicken embryogenesis and in the developing wing

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ABSTRACT Previously, we isolated an avian protein which we named retinoic acid induced heparin binding factor (RIHB). RIHB is a 121 amino acid secreted polypeptide, rich in basic and cysteine residues (Vigny *et al., Eur. J. Biochem. 186:* 733-740, 1989). Northern blot analysis indicates that the RIHB gene is transiently expressed during embryogenesis (Urios *et al., Biochem. Biophys. Res. Com. 175:* 617-624, 1991). Here we present an investigation of RIHB expression during early chicken embryogenesis by *in situ* hybridization and immunofluorescence studies. In the 3-day embryo (stage 20-21), the RIHB transcript is observed throughout the embryo, with the notable exception of the neural tube. At this stage the protein can be visualized in almost all of the basement membranes and around many types of cells. The localization of the RIHB protein does not strictly parallel that of its messenger. Between days 3 and 11 we focused our attention on wing development. The level of both the mRNA and protein decreases during this period but the disappearance is not uniform. The transcript becomes progressively restricted to epithelia and regions surrounding the forming cartilage. In contrast to the transcript, the protein accumulates in the epithelial basement membrane and, interestingly, in the central part of the embryonic cartilage (diaphysis) but not in the distal parts (epiphysis). These data are discussed in relation to the putative role(s) of RIHB in development.

KEY WORDS: *chick embryo, retinoic acid, heparin binding, basement membrane, growth and differentiation factors, wing development*

Introduction

Retinoids, which are derivatives of vitamin A (retinol), are involved in diverse processes of development and appear to be key modulators of embryonic differentiation and development. For example, retinoic acid (RA) is strongly implicated in limb morphogenesis, since local application to the chick wing results in alterations in limb pattern formation such as mirror image duplication (Tickle *et al.*, 1982). Although some controversy persists, (Noji *et al.*, 1991) the data support a role for RA in limb patterning. The identification of retinoic acid receptors (RARs and RXRs) (Petkovich *et al.*, 1987; Benbrook *et al.*, 1988; Krust *et al.*, 1989; Zelent *et al.*, 1989; Mangelsdorf *et al.*, 1991; Leid *et al.*, 1992), which belong to the superfamily of nuclear receptors and act as ligand-inducible transcription enhancing factors, has greatly advanced the understanding of how retinoids may control many aspects of embryonic development (Tabin, 1991; Mendelsohn *et al.*, 1992 and references therein).

We have previously purified to homogeneity a heparin-binding protein from chicken embryos which appears, at least in the embryonic eye, to be mainly localized within basement membranes (Vigny *et al.*, 1989). Since synthesis of this protein is controlled by retinoic acid in certain cellular systems, it was designated "retinoic acid-induced heparin-binding" or RIHB (Raulais *et al.*, 1991). A cDNA clone was isolated (Urios *et al.*, 1991) and the deduced sequence predicts that RIHB contains 121 amino-acids and is very rich in basic amino acids and cysteine residues. Northern blot analysis of RNA extracted from whole embryos indicates that the level of RNA expression is high during early embryonic development (days 2-4) then decreases, and is barely detectable at day 14 (Duprez *et al.*, 1991; Urios *et al.*, 1991). Thus, RIHB is a marker of early embryonic development and is controlled by RA.

RIHB belongs to a new family of heparin binding proteins which have interesting patterns of expression during development. The MK (midgestation-kidney) gene was identified by the differential hybridization technique. A cDNA clone was isolated, corresponding to an RNA which is transiently expressed in the early differentiation stage of embryonic carcinoma HM1 cells induced by retinoic acid (Kadomatsu *et al.*, 1988). High expression of the MK gene occurs during the midgestation period of mouse embryogenesis. Furthermore, the amino acid sequence of RIHB shows 65% homology with that of MK. These data suggest that MK is the mammalian

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0214-6282/93/$03.00

Printed in Spain

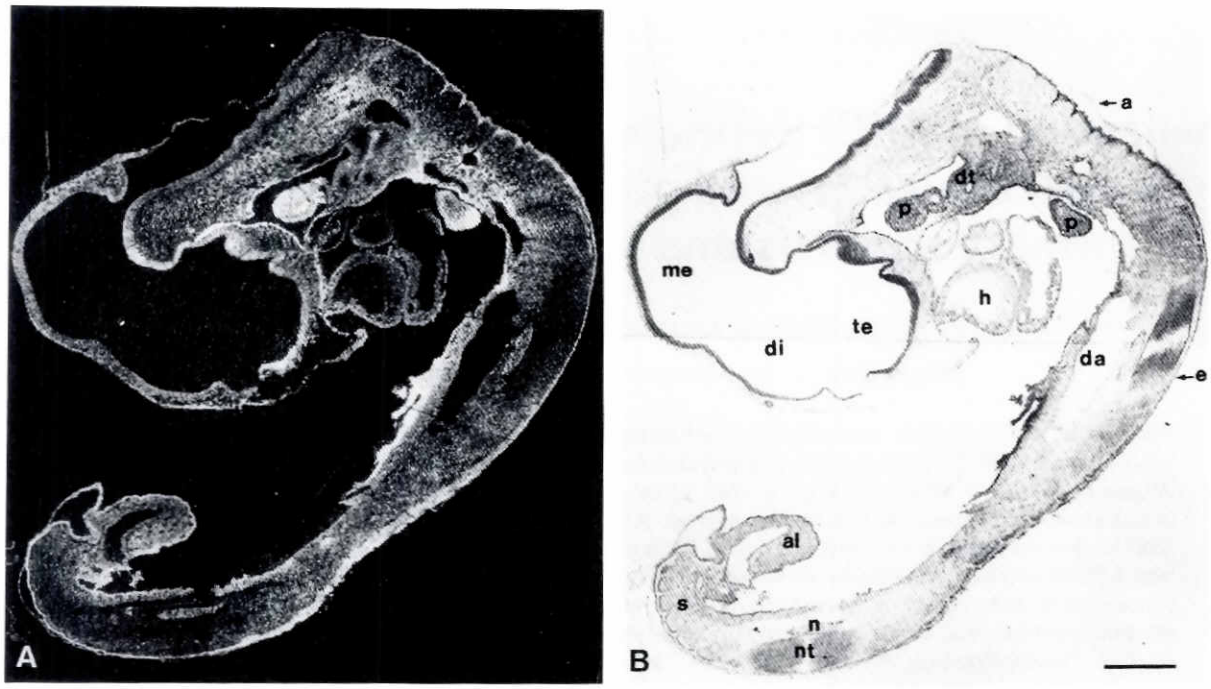

Fig. 1. RIHB mRNA expression in a 3-day (stage 20-21) chick embryo. *RIHB antisense probe was hybridized to a sagittal section of a 3-day embryo* **(A)**. *Several photos of dark field photomicrograph were combined to show the whole embryo. The bright field photomicrograph* **(B)** *represents the same section stained with hematoxylin. The exposure time was 10 days. a, amnios; al, allantoid; da, dorsal aorta; di, diencephalon; dt, digestive tract, e, ectoderm; h, heart; me, mesencephalon n, notochord; nt, neural tube; p, pulmonary bud; s, somites; te, telencephalon. Scale bar, 600 μm.*

counterpart of RIHB, but this point remains to be demonstrated definitively. The second member of the family, which we will refer to in this paper as heparin-binding-growth-associated molecule, HBGAM (Merenmies and Rauvala, 1990), has received several names, reflecting the diverse laboratories where this molecule was identified (listed in Böhlen and Kovesdi, 1991). HBGAM shows 50% sequence homology with MK and RIHB and is expressed later in development than these molecules. For example, it is primarily expressed during the perinatal period of rat development in the brain and muscle. The HBGAM gene is activated in cultured teratocarcinoma cells undergoing RA induced differentiation (Kretschmer *et al.*, 1991). The biological properties of these proteins are still poorly understood. Embryonic neurons and PC12 cells extend neurites when grown in cultured dishes coated with HBGAM (Rauvala, 1989; Bohlen *et al.*, 1991), MK (Muramatsu and Muramatsu, 1991) or RIHB (Raulais *et al.*, 1991). Given the large amounts of lysine residues present in these proteins, this observed effect on neurite outgrowth could be analogous to a polylysine effect. MK and HBGAM have also been suggested to be mitogenic but contradictory results have been reported. Possible explanations for these discrepancies may include a contamination of MK/HBGAM samples with FGF-like or other mitogenic substances (for a review see Kovesdi and Bohlen, 1991). However, it has recently been reported that recombinant HBGAM (Fang *et al.*, 1992) and recombinant MK (Nurcombe *et al.*, 1992) produced by transfection of mammalian cells are mitogenic. Whatever the case, the transient expression of MK and HBGAM and their inducibility by retinoic acid *in vitro* strongly suggest that they play a role in embryonic development. Knowledge of the expression pattern of these proteins and their mRNA in developing tissues would contribute to our understanding of the roles of these molecules. The localization of the HBGAM transcript has been analyzed in the developing and adult rat tissues (Vanderwinden *et al.*, 1992). The precise localization of the MK gene expression has been analyzed during mouse embryogenesis by *in situ* hybridization (Kadomatsu *et al.*, 1990), but no information is available concerning the MK protein localization.

In this report we describe at stages 20-21 of chick embryonic development both the RIHB gene expression (mRNA) analyzed by *in situ* hybridization and the localization of the RIHB protein analyzed by indirect immunofluorescence studies using specific monoclonal antibodies. At later stages, i.e. between day 6 and day 15 of embryonic development, we focus our attention on the development of the wing, since RA is involved in limb morphogenesis. The expression of both the RIHB mRNA and protein in developing cartilage was analyzed in detail, because the spatio-temporal pattern of expression of the messenger and of the protein were different in this tissue. We also compared the pattern of expression of RIHB found in the diaphyseal region of the forming cartilage between days 6 and 11 with those of type II and type X collagen. The data are discussed in relation to the putative role(s) of RIHB in cartilage differentiation and development.

Results

Distribution of the RIHB transcript and protein in 3-day-old chick embryos

We analyzed by *in situ* hybridization the distribution of the RIHB transcript in 3-day-old embryos (stage 20-21). (Figs. 1, 2). Using the antisense probe it appeared that the RIHB mRNA was strongly expressed throughout the embryo (Figs. 1A, 2A). However, the

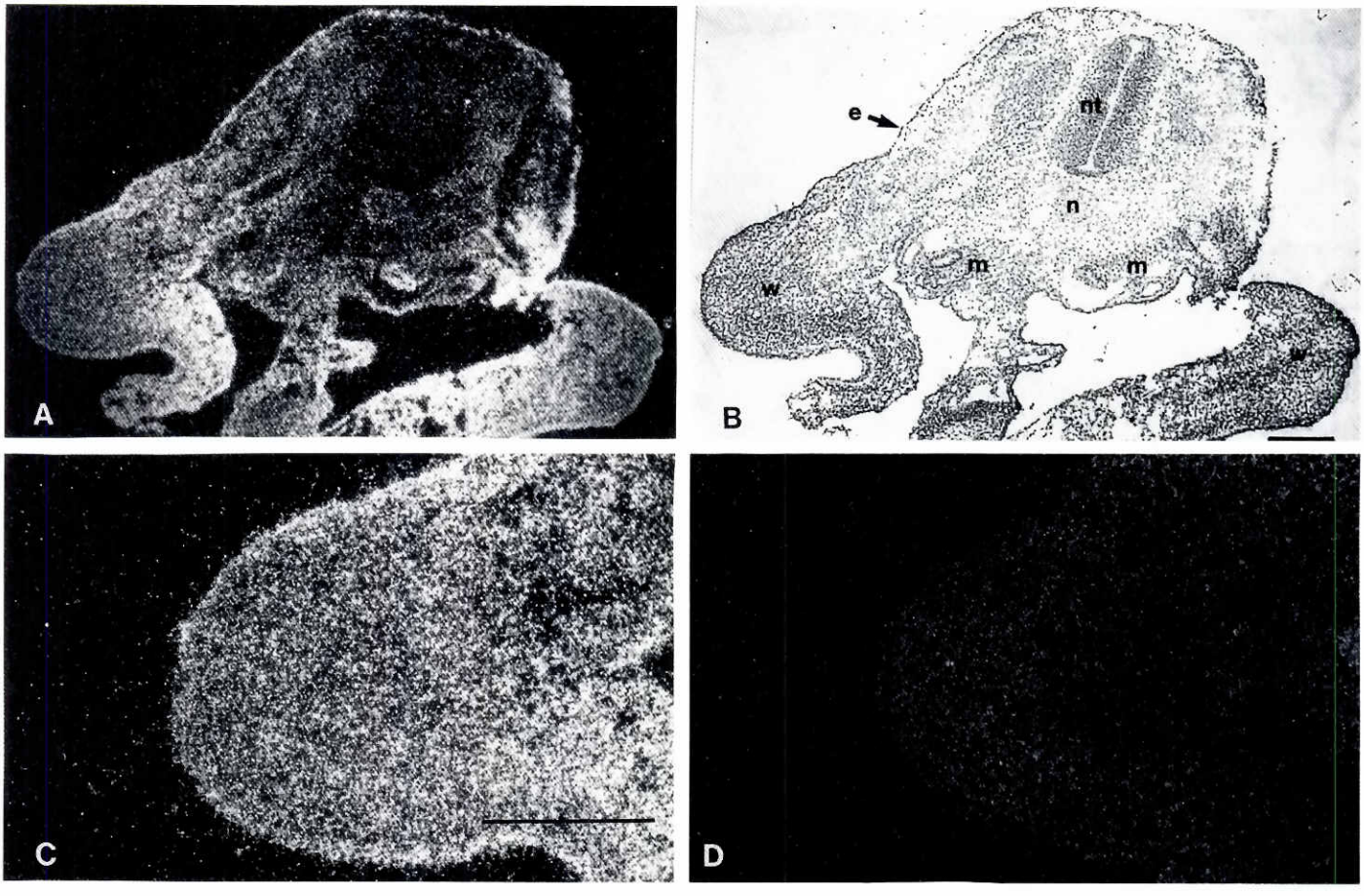

Fig. 2. RIHB mRNA expression in a 3-day (stage 20-21) chick embryo. *RIHB antisense probe was hybridized to a transverse section at the wing level of a 3-day embryo* **(A)**. *The bright field photomicrograph represents the same section stained with hematoxylin* **(B)**. *Higher magnification (3-fold) of the wing hybridized with the antisense probe* **(C)** *and with the sense probe* **(D)**. *The exposure time was 10 days. e, ectoderm; m, mesonephros tubules and ducts; n, notochord; nt, neural tube; w, wing bud. Scale bars, 200 μm.*

expression was not uniform among the different embryonic tissues. The ectoderm, limb mesenchyme, chord, pulmonary bud, digestive tract, the mesonephros ducts and tubules and the amnios exhibited a stronger accumulation of the messenger than other tissues. It is also noteworthy that the presence of the mRNA was barely detectable in the neural tube, whereas it was clearly present in the telencephalon, diencephalon and mesencephalon. The Rathke's pocket is also stained but the epiphysis is not. Using the sense RIHB probe as a control, few silver grains were detected (Fig. 2D).

We analyzed the distribution of the RIHB protein by immunofluorescence using monoclonal antibodies (Fig. 3). Specific polyclonal antibodies to RIHB gave identical results (data not shown). Like the mRNA, the protein was also widely distributed (Fig. 3A). As previously demonstrated in the embryonic eye (Vigny *et al.*, 1989), the protein was present in many basement membranes of the 3-day-old embryo. For example, the basement membranes located beneath the ectoderm and surrounding the notochord, the dorsal aorta and the mesonephros tubules and ducts are clearly and uniformly stained. The case of the neural tube basement membrane is also worth pointing out, because RIHB was more concentrated in the dorsal part of this structure. Besides the localization in the basement membranes, an intense pericellular staining can be visualized around many cell types. This staining is particularly obvious around the cells of the limb mesenchyme. Molecular cloning of RIHB indicated the presence of a signal peptide sequence typical of secreted proteins (Urios *et al.*, 1991). Thus, RIHB is secreted and appears to be associated to the plasma membranes of cells or to the basement membranes.

RIHB clearly accumulates in the basement membrane surrounding the notochord, suggesting that the protein is synthesized, secreted and then concentrated in the basement membrane. The same process probably occurs in the basement membrane surrounding mesonephros tubules and ducts and in the basement membrane underlying the ectoderm. The protein was also only weakly detected in the neural tube, which is in agreement with the *in situ* hybridization data. However the dorsal part of the neural tube is richer in RIHB than the ventral part, as pointed out for the basement membrane surrounding this structure. The use of negative hybridoma supernatant revealed no fluorescence (Fig. 3B).

RIHB expression becomes restricted during wing morphogenesis

We focused on wing development for three reasons. First, we detected a high level of expression of RIHB mRNA and protein in the wing bud at day 3 (Figs. 1,2,3). Second, retinoids are known to be

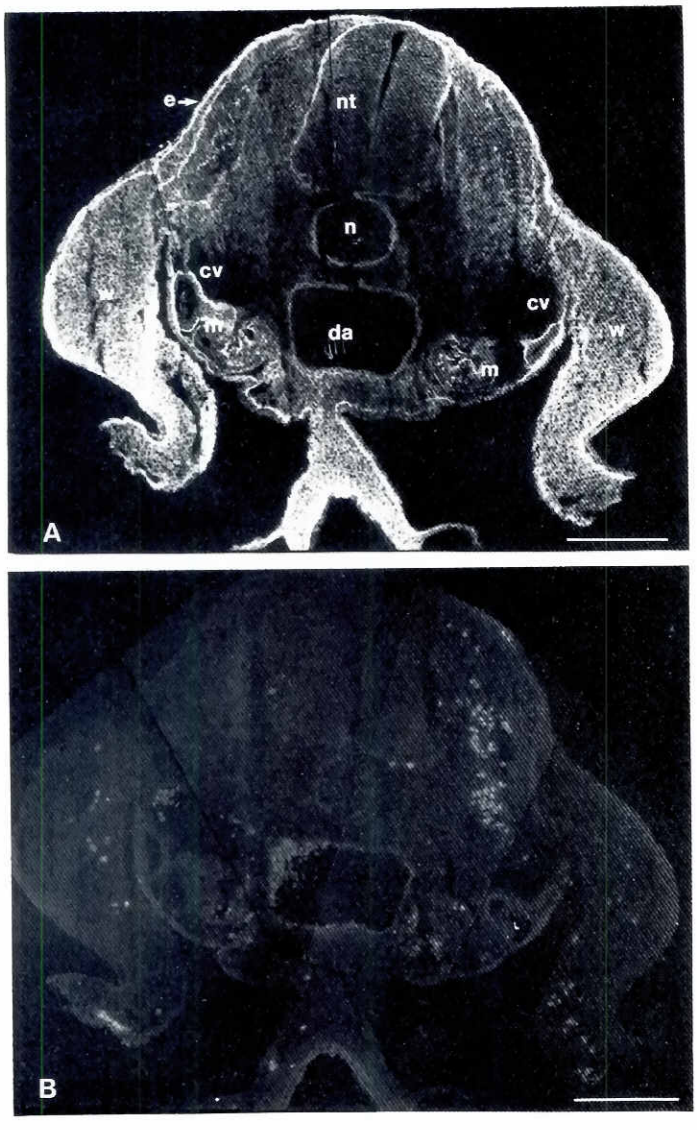

Fig. 3. Indirect immunofluorescence staining of transverse sections at the wing level from 3 day (stage 20-21) chick embryo, *labeled with monoclonal antibody against RIHB and FITC-conjugated rabbit anti mouse γ-globulin* **(A)**, *and labeled with a monoclonal antibody which does not crossreact with RIHB* **(B)**. *The autofluorescence of blood cells is a nonspecific signal. cv, cardinal veins; da, dorsal aorta; e, ectoderm; m, mesonephros tubules and ducts; n, notochord; nt, neural tube; w, wing bud. Scale bars, 200 μm.*

involved in limb development (for a review see Tabin, 1991). Third, RA modulates the morphology of chondrocytes (Solursh and Meier, 1973) and affects the expression of genes in chondrocytes *in vitro* (Horton and Hassel, 1986; Pacifici *et al.*, 1991).

Distribution of the RIHB transcript

The localization of the RIHB transcript in the 3-day-old limb bud can be seen in Fig. 2C. The transcript was uniformly distributed throughout mesenchyme and ectoderm, with a higher expression in the ectoderm. At day 6, the expression in the wing is more restricted (Fig. 4). RIHB mRNA was still strongly expressed in the epidermis but was barely detectable in many areas of the mesenchymal/muscular tissues. It was highly concentrated in the mesenchymal region surrounding the forming cartilage. The localization of cartilage-specific proteoglycan was revealed by staining with alcian blue (Fig. 4D). Hybridization with the RIHB probe appeared to be greater in the distal parts (the future epiphyseal regions) of the developing ulna and digit III than in the central part (future diaphyseal region). At this stage there is no perichondrium in the epiphyseal region and the chondrocytes merge with the surrounding mesenchyme (Rooney *et al.*, 1984). In the 8-day-old embryonic wing (Fig. 5) the expression was more clearly restricted to the regions listed above. The messenger still accumulated in the epidermis and in the epiphyseal region of the developing cartilage (Fig. 5A). Furthermore, in the diaphyseal regions of the scapula, humerus, ulna, digit III and digit IV, the messenger appeared concentrated in the perichondrium surrounding this region. At day 11 expression could still be visualized in the epidermis but it was much less intense than that observed at day 8, and the presence of the RIHB transcript was not detected elsewhere in the wing (data not shown). At day 15 no transcript could be detected by *in situ* hybridization in the epidermis.

In order to quantify the RIHB mRNA content of the wing during its morphogenesis we analyzed RIHB gene expression by Northern Blot analysis (Fig. 6). The RIHB transcript was abundant in 3-day-old embryonic wing and progressively decreased until day 11. At day 14 almost no transcript was observed in the wing (data not shown).

We also attempted to quantify the expression of the messenger detected by *in situ* hybridization in the different tissues at different ages. This study was done by counting the number of silver grains per cell, the background being determined on the same tissues from the sense hybridization experiments. The study was performed for the epidermis at days 3, 6, 8 and 11; and for the presumptive ulna at day 3 or for the ulna (perichondrium and core) at days 6, 8 and 11 (Fig. 7). The data obtained were consistent with those described above; the expression diminishes progressively during development. Furthermore, at days 6, 8 and 11, the expression of the messenger in the epidermis was very high compared to the expression in the perichondrium and in the cartilage core; this latter being close to the background. This point is important when considering the different localization of the protein and the messenger (see below and discussion).

The localization of the protein is different from that of the mRNA in embryonic cartilage

The expression of the transcript during wing development led us to investigate that of the protein in the developing cartilage. We will concentrate on the ulna (see below) after drawing attention to several noteworthy aspects of the expression of RIHB in the epidermis and in the mesenchyme/premuscular tissue during wing development.

The distribution of the RIHB protein in the 3-day-old embryonic wing bud, as evaluated by staining with specific antibodies, was almost uniform (Fig. 3A). As previously indicated, RIHB was present in the basement membrane underlying the epidermis and concentrated around the mesenchymal cells of the wing. At day 6 the protein was still accumulated in the basement membrane (Fig. 8) although the staining around the mesenchymal/muscular cells was greatly reduced but always above the background. Thus, we consider that RIHB was still present at a low level around mesenchymal cells. At day 8 no RIHB could be detected in the mesenchyme (Fig. 9). The progressive disappearance of the protein from the surface

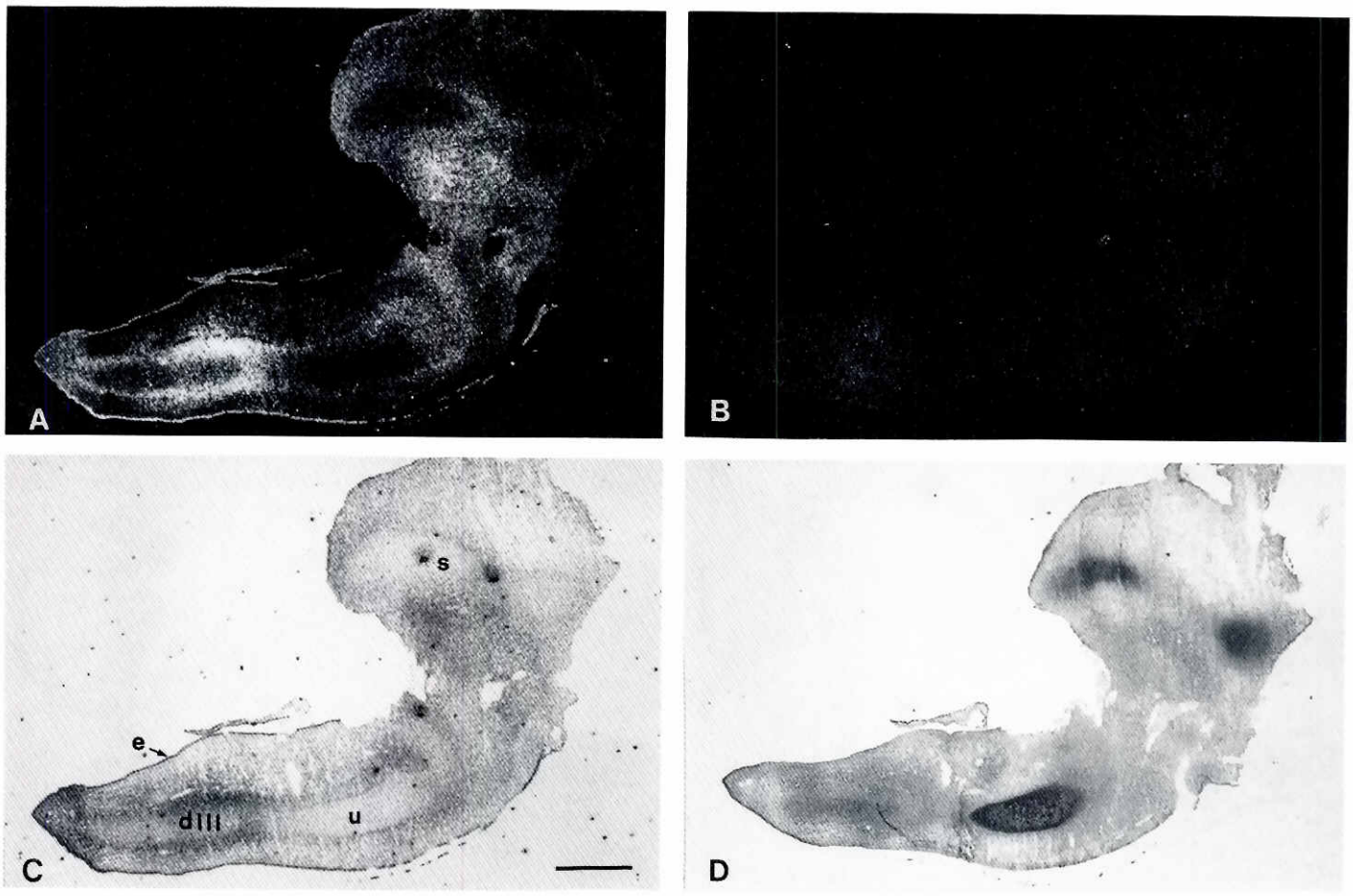

Fig. 4. *In situ* hybridization of RIHB mRNA in the wing bud at day 6 (stage 28). *RIHB antisense* **(A)** *and sense* **(B)** *probes were hybridized to longitudinal sections of wing bud at day 6. Bright field photomicrographs represent the antisense section stained with hematoxylin* **(C)** *and the adjacent section stained with alcian blue* **(D)**. *dIII, digit III core; e, epidermis; s, scapula; u, ulna core. Scale bar, 600 μm.*

of the mesenchymal cells occurred between days 3 and 6, and was complete at day 8. At this age RIHB was always present in the epidermal basement membrane but the immunofluorescence staining was reduced compared to that observed at day 6 (Fig. 9A compared to Fig. 8A). RIHB was present in this structure until day 13-14 (data not shown); again a progressive disappearance of RIHB was observed but occurring later than in the mesenchyme. In addition, between days 13-14 the protein was still detectable in the basement membrane whereas the transcript had almost completely disappeared from the epidermal cells.

The fluorescence observed in the cartilage of the 6-day chick ulna is worthy of comment (Fig. 8A). For all immunohistochemical analyses, cartilage sections were predigested with hyaluronidase to remove cartilage proteoglycans (Von der Mark, 1980; Schmid and Linsenmayer, 1985). In the absence of this treatment the staining was much less intense, suggesting that some of the RIHB epitopes were masked or trapped in the cartilaginous matrix. At this stage the ulna can be divided into three zones. In the epiphyseal region the cells are rounded and actively dividing. The rounded cells progressively flatten to constitute the intermediate flattened zone. In the central diaphyseal region the cells undergo hypertrophy characterized by a massive matrix secretion (Fell, 1925; Rooney *et al.*, 1984). The RIHB protein was strongly accumulated in the matrix surrounding the chondrocytes in the region of the future diaphysis and reduced in the future epiphyseal region where the cells are rounded. The perichondrium is also stained. Thus, at this age there is a gradient of RIHB from the central part to the distal parts of the ulna. In comparison, type II collagen, a marker of the cartilage matrix (Fig 8B), was uniformly distributed in the cartilage, as has been previously described (Von Der Mark *et al.*, 1976a,b). Thus, the distributions of collagen II and RIHB were different. In conclusion, at day 6 the protein is found in the perichondrium and inside the cartilage matrix, whereas the transcript is observed primarily around the ulna core.

At day 8 the patterns of expression of RIHB and type II collagen were similar to those obtained at day 6 (Fig. 9). The gradient of RIHB is perhaps slightly more pronounced but the protein is still present in the epiphyseal region. In addition, the perichondrium was positively stained with the RIHB antibody and not with the type II collagen antibodies. No type X collagen could be visualized at this stage (data not shown), confirming previous reports (Capasso *et al.*, 1984; Schmid and Linsenmayer, 1985). At day 11, type II collagen is expressed almost uniformly all along the ulna (data not shown). The distribution of RIHB is illustrated in Fig. 10A. The intensity of the

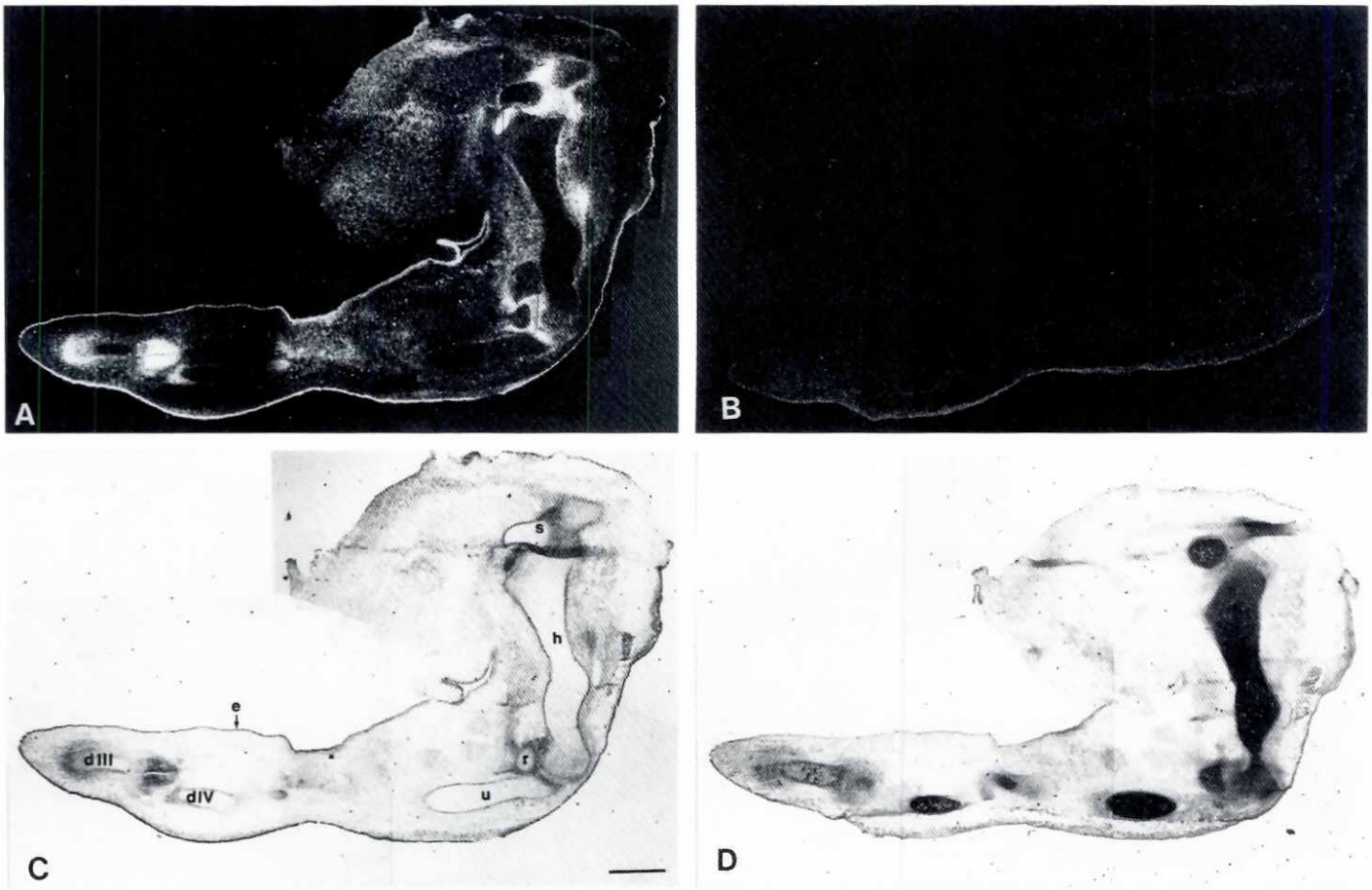

Fig. 5. *In situ* hybridization of RIHB mRNA in the wing at day 8 (stage 34-35). *RIHB antisense* **(A)** *and sense* **(B)** *probes were hybridized to longitudinal sections of wing at day 8. Bright field photomicrographs represent the antisense section stained with hematoxylin* **(C)** *and the adjacent section stained with alcian blue* **(D)**. *dIII, digit III ; dIV, digit IV; e, epidermis; h, humerus; r, radius epiphysis; s, scapula; u, ulna. Scale bar, 600 μm.*

staining in the diaphyseal region is not diminished but the protein is now completely absent from the epiphysis. Thus, RIHB was restricted to the region of the flattened and hypertrophic chondrocytes and still present in large amounts. In contrast, the level of the messenger in the cartilage and surrounding tissues detected by *in situ* hybridization and Northern Blot analysis was greatly reduced at this stage (Figs. 6,7). As previously described (Schmid and Linsenmayer, 1985), type X collagen was expressed in the central part of the cartilage corresponding to the region of the diaphysis containing hypertrophic chondrocytes (Fig. 10B). Comparison of the staining patterns of type X collagen, RIHB and collagen type II is interesting. Type X collagen was found in the central part of the bone, RIHB extended from this region, and type II collagen was found throughout the ulna. Thus, during cartilage growth RIHB progressively disappears from the epiphysis but is always present in the zone of flattened cells and strongly accumulated in the central diaphyseal region.

At day 13, the fluorescence obtained with the specific RIHB antibodies is greatly diminished compared with that obtained with type II and X collagen antibodies (data not shown). At day 15, no RIHB staining could be observed in the different parts of the forming bone (data not shown).

Discussion

Early embryo

The pattern of RIHB gene expression in the early chicken embryo (stage 20-21) was examined in the present investigation. Extensive expression of the transcript was observed throughout the embryo except in the neural tube, where hybridization was less intense. The RIHB gene appears to be transiently expressed in almost all cell lineages. This distribution is reminiscent of that previously described for the MK gene during the midgestation period of the mouse (Kadomatsu *et al.*, 1990). In particular, the sites where the RIHB messenger was more intensively expressed in the chick embryo (day 3) are similar to those observed for the MK gene in the 11-day-old mouse embryo. In addition to the evidence described in the introduction, these data support the hypothesis that MK is the mammalian counterpart of RIHB. Furthermore, these areas include the regions where secondary embryonic induction is prominent (Gilbert, 1988). Examples of the best studies of secondary induction are those involving the interactions of epithelial sheets with adjacent mesenchymal cells. These are called epithelio-mesenchymal interactions. Regions where this mechanism is thought to operate are cutaneous structures, the limb, the lung and

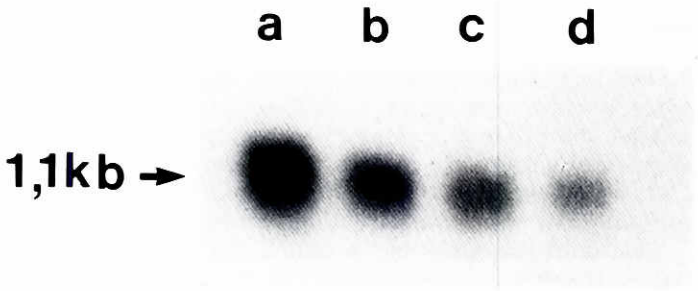

Fig. 6. Northern blot analysis of the RIHB transcript expression in the whole wing during embryonic development. *Tracks contain 10 μg of total RNA isolated from 4-day wing bud* **(a)**, *6-day wing bud* **(b)**, *8-day wing* **(c)**, *11-day wing* **(d)**. *The filter was hybridized with ^{32}P-labeled RIHB cDNA (780 bp). Size of transcripts is indicated in kilobases. Exposure time was 24 h.*

the kidney. The epithelial component is separated from the mesenchyme by the basement membrane. The fact that RIHB is transiently present in the basement membrane of these tissues during the critical phases of secondary induction suggests that the RIHB plays a fundamental role in the differentiation of a wide variety of cell types.

Like the transcript, the RIHB polypeptide was widely distributed among the embryonic tissues. We previously analyzed in detail the localization of RIHB during ocular development. In the eye the protein appeared mainly associated with the multiple basement membranes present in this organ (Vigny *et al.*, 1989). The staining pattern in the whole 3-day-old embryo indicates that RIHB is present in almost all the basement membranes and also around many cell types at this stage. We previously demonstrated that RIHB, as a heparin binding protein, could interact with the heparan sulfate proteoglycan present within the basement membranes (Vigny *et al.*, 1989). This proteoglycan is presently called perlecan (Noonan *et al.*, 1991). It is tempting to suggest that RIHB also interacts with heparan sulfate proteoglycans such as syndecans present on the plasma membrane (Saunders *et al.*, 1989). When the limb bud is just apparent, syndecan 1 is detected on cells throughout the limb region including both ectodermal and mesenchymal components. Thus, at this stage syndecan 1 codistributes with RIHB. Later syndecan 1 decreases in the precartilage condensation (Solursh *et al.*, 1990), whereas syndecan 3 appears in the chondrogenic core (Gould *et al.*, 1992). The affinity of RIHB for different members of this growing family of heparan sulfate proteoglycans could be informative of the biological activity of this protein. The example of the notochord (see Results) indicated that some cells may be devoid of heparan sulfate proteoglycans or other binding sites on their surface. If this is the case, the secreted RIHB may accumulate into the basement membrane surrounding this structure. The same process could be valid for all basement membranes, including the epidermal basement membrane. Thus, basement membranes could act as a reservoir for RIHB, as has been proposed for basic fibroblast growth factor (bFGF) (Vlodavsky *et al.*, 1987).

Limb morphogenesis and cartilage formation

Limb formation has been extensively studied in the chick. The apical ectodermal ridge (AER) is responsible for the sustained outgrowth and development of the limb (Saunders, 1948). As cells leave the progress zone (PZ), they have their proximal-distal values specified and differentiate into cartilage (Saunders, 1948; Summerbell *et al.*, 1973). In contrast, the zone of polarizing activity (ZPA) organizes the antero-posterior axis of the developing limb and releases a morphogen (Tickle *et al.*, 1975), which could be trans-retinoic acid (Tickle *et al.*, 1982, 1985; Thaller and Eichele, 1987). RIHB (mRNA and protein) expression was uniform in the limb at day 3. Thus, there is no straight forward relationship between the expression of RIHB and the control of the antero-posterior and proximo-distal axis during the formation of the limb.

During limb formation the expression of the RIHB transcript became progressively more restricted. RIHB mRNA disappeared from the mesenchymal/muscular tissues at day 6-8 and was expressed only in the epithelium and around the forming cartilage. The messenger could still be visualized in the epidermis until day 13. It should be noted that the RIHB protein localization did not follow this time course, since it could be visualized in the basement membrane underneath the epidermis until day 15. Thus, there is a delay of several days between the attenuation of the synthesis of the protein (detected by the presence of the transcript) and the disappearance of the protein stored (and protected) in the basement membranes.

The mode of expression of the RIHB gene during cartilage formation is worth noting. At day 6, the transcript was primarily visualized in the future epiphyseal region, i.e. the region where the cartilage cells merge with surrounding mesenchyme. The chondroblasts are derived from a mesenchymal precursor, and cartilage differentiation involves a quantitative increase in matrix synthesis, and a qualitative change in the pattern of gene expression (Vertel and Dorfman, 1978; Von Der Mark, 1980; Hayashi *et*

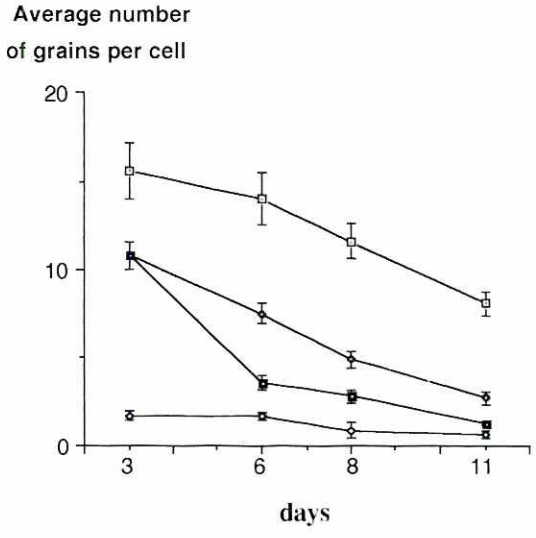

Fig. 7. Quantification of the level of expression of RIHB mRNA in the developing wing using image analysis after *in situ* hybridization. *Following 10 day exposure, silver grains were counted over the ectoderm (—□—) at 3, 6, 8 and 11 days, over the presumptive region of ulna at 3 days and over the ulna core (—■—) and perichordium (—◆—) at 6, 8 and 11 days. Quantification of the sense signal, i.e. the background, is represented by (—◇—). Silver grains per cell were plotted against time in days. Each point represents the average number of grains per cell in 920 μm² determined from 2 to 4 serial sections. Vertical lines through each point represent the standard deviations.*

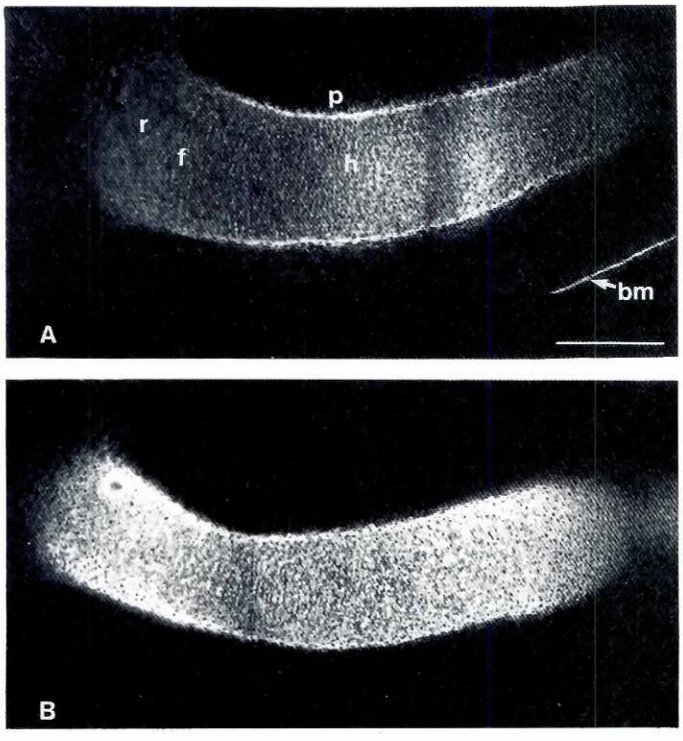

Fig. 8. Serial longitudinal sections of developing ulna at day 6 (stage 28) *stained with anti-RIHB monoclonal antibody* **(A)**, *stained with anti collagen type II polyclonal antibody* **(B)**. *bm, epidermal basement membrane; f, flattened chondrocytes; h, hypertrophic diaphyseal chondrocytes; p, perichondrium; r, rounded epiphyseal chondrocytes. Scale bar: 200 μm.*

al., 1986; Kosher *et al.*, 1986). Thus, the differentiation of the chondrocytes was concomitant with the synthesis of RIHB mRNA. In contrast, the expression of the transcript in the center of the forming cartilage (diaphysis) was weak, whereas the protein was strongly accumulated in the matrix surrounding the chondrocytes in this area. Thus the localizations of the messenger and of the protein were very different. One can ask: what is the origin and the half-life of the protein in the diaphyseal cartilage matrix? We cannot exclude that the chondrocytes located in this area synthesize their own RIHB but our data indicated a very low level of transcript in this region. Thus, it is tempting to suggest that the protein is synthesized and secreted in the future epiphyseal region, and then accumulated in the diaphyseal region. *In situ* hybridization data support this interpretation. In the epiphyseal region the messenger is strongly expressed at day 6, reduced at day 8 and absent at day 11 in the ulna. This time course of RIHB mRNA expression is probably the cause of the gradient of the protein found in the ulna at days 6, 8 and 11. Thus at day 11 the RIHB concentrated in the center of the diaphyseal region would have been synthesized several days before. This hypothesis implies, of course, a long half-life of the protein in the cartilage matrix.

RIHB disappears around day 13 and therefore it is not involved in the transition process of the cartilage into bone or in the bone formation. It is interesting to note that HBGAM, also called OSF1 for osteoblast specific factor (Tesuka *et al.*, 1990) has been suggested as playing a fundamental role in bone formation. As pointed out in the introduction, HBGAM/OSF1 is expressed later in development. Thus, it would be interesting to analyze the expression of the second member of this family during bone formation because it could play a role in this process similar to RIHB in cartilage formation. In addition, the growth of the mature cartilaginous growth plate is roughly similar to that of embryonic cartilage in that it has separate zones of proliferation and cartilage hypertrophy. No RIHB has been detected in the mature cartilaginous growth plate.

What is the biological role of RIHB in the diaphyseal region (regions of flattened and hypertrophic cells)? The strong accumulation of RIHB in the diaphyseal region could reflect the role played by this protein in cell differentiation processes occurring in this specific area. In the diaphyseal region, the differentiation of chondrocytes into hypertrophic chondrocytes is characterized by the production of type X collagen (Schmid and Linsenmayer, 1983, 1985). The cartilage at this region will go on to be replaced by bone tissue. The wave of cell hypertrophy that progresses from the diaphysis towards the epiphysis implies a gradient of unknown factors with the high point in the center (Wolpert and Stein, 1984). Thus, hypertrophy begins at the center and proceeds in both direction to the epiphysis down the gradient(s). The graded distribution of RIHB before the onset of the hypertrophy process strongly suggests that this protein may be linked to this differentiation process.

However, a second putative role for RIHB can be suggested. The cell division occurring in the epiphysis probably requires the presence of mitogenic growth factors such as FGFs. It has been clearly established that basic FGF is a potent mitogen for chondrocytes *in vitro* (Kato and Gospodarowicz, 1984) and promotes cartilage repair *in vivo* (Cuevas *et al.*, 1988). Other heparin binding growth factors such transforming growth factor ß (TGFß) and the related bone morphogenetic proteins (BMP) are also involved in cartilage growth and differentiation (see Hill and Logan, 1992 for a review). It is now established that cell surface heparin-like molecules are necessary for the binding of basic FGF to its high affinity tyrosine kinase receptors (Yayon *et al.*, 1991). Taking into account the large amount of RIHB present in the central part of the cartilage, one can propose that FGFs and/or other heparin binding growth factors could not bind to the heparan sulfate chains blocked by RIHB. Thus RIHB could also act as a competitor of heparin binding growth factors for their so-called low affinity binding sites, thus inhibiting their biological activities. Some support for this notion comes from reports showing that recombinant bacterial MK protein prevents infection by herpes simplex virus by competing at the heparan sulfate sites on syndecans which serve as the cellular receptors for the proteins of the viral envelope (Ostrander *et al.*, 1992).

In conclusion, the expression of RIHB during embryonic development appears to be linked to cell differentiation processes. In addition, it could modulate the biological activities of heparin binding growth factors. These two putative, and probably linked, roles of RIHB are currently under investigation in the cartilage and also in other differentiating tissues where RIHB is transiently expressed.

Materials and Methods

Probe preparation

A RIHB cDNA clone of 754 bp was subcloned in Bluescript. This cDNA clone contains the complete coding region (426 bp) and the 3' untranslated region. The plasmid template was linearized with BamH I (to generate Antisense probe) and with Kpn (sense probe). Antisense and sense

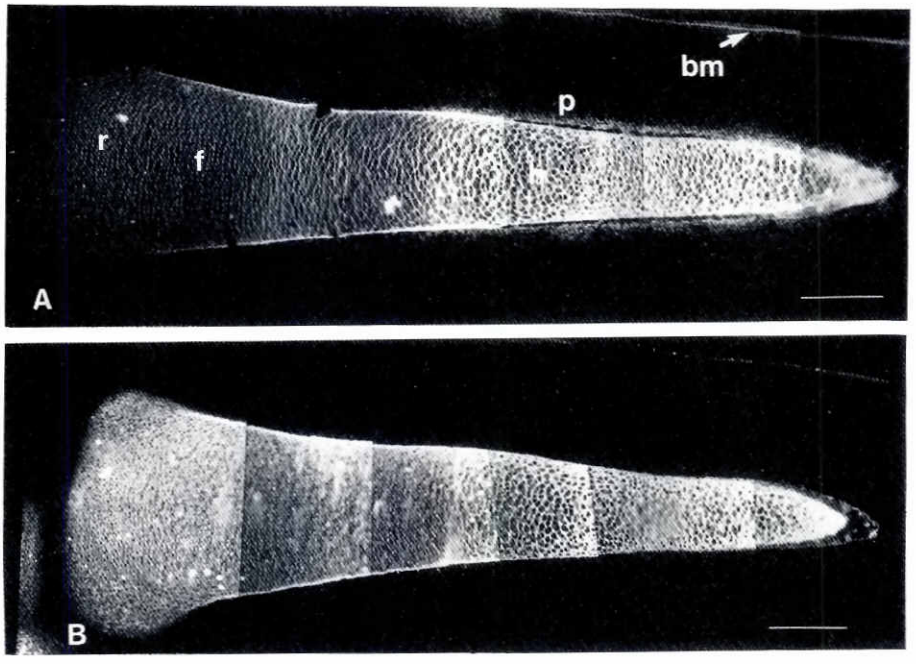

Fig. 9. Serial longitudinal sections of developing ulna at day 8 (stages 34-35) *stained with anti-RIHB monoclonal antibody* **(A)**, *and stained with anti collagen type II polyclonal antibody* **(B)**. *bm, epidermal basement membrane; f, flattened chondrocytes; h, hypertrophic diaphyseal chondrocytes; p, perichondrium; r, rounded epiphyseal chondrocytes. Scale bar, 200 mm.*

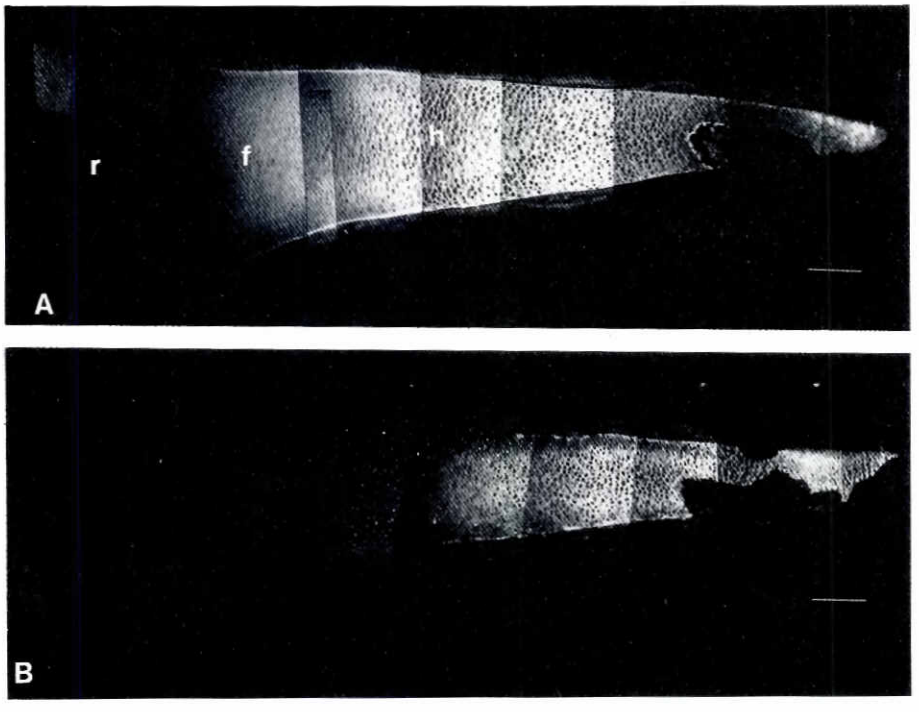

Fig. 10. Serial longitudinal sections of the developing ulna at day 11 (stage 37) *stained with anti-RIHB monoclonal antibody* **(A)**, *and stained with anti collagen type X polyclonal antibody* **(B)**. *bm, epidermal basement membrane; f, flattened chondrocytes; h, hypertrophic diaphyseal chondrocytes; p, perichondrium; r, rounded epiphyseal chondrocytes. Scale bars, 200 μm.*

transcripts were synthesized by incubation of 1 μg of the appropriate linearized template DNA at 37°C for 1 h in Tris-HCl pH 7.5 containing 10 mM dithiothreitol, 0.4 mM ATP, GTP, CTP and 0.02 mM [α-^{35}S] UTP 800 Ci/mmole (Amersham), 1 unit/μl RNAse Guard (BRL) and 1 unit/μl T7 (to generate the probe Sense) or T3 (for the Antisense probe) RNA polymerases (BRL). Templates were digested by incubation with 0.1 unit/ml DNAse RQ1, RNAse-free, (Promega) at 37°C for 30 min. The probes were reduced in size (200 bp) by alkaline hydrolysis and were incubated with 0.06 M Na_2CO_3 and 0.04 M $NaHCO_3$ containing 10 mM dithiothreitol at 60°C for 30 min. Labeled probes were concentrated by ethanol precipitation.

In situ hybridization

The method used for hybridization was a modification of that described by Cox *et al.* (1984) and Simmons *et al.* (1989).

Tissue preparation and pretreatment

Chick embryos or tissues were removed at different stages according to Hamburger and Hamilton (1951). They were mounted unfixed in tissue-Tek OCT medium (Miles laboratories), rapidly frozen in liquid nitrogen, and sectioned at -20°C. Ten micron thick sections were fixed in 4% paraformaldehyde for 30 min and washed twice for 3 min in fresh PBS.

Prehybridization treatment of tissue sections was done by immersing the slides in 0.01% Triton X-100 for 1.5 min, then washing (2 times, 3 min) in PBS and incubating for 7.5 min with predigested proteinase K at final concentration of 1 μg/ml in 50 mM Tris-HCl pH 7.4, 5 mM EDTA, then washing with PBS containing 2 mg/ml glycine (twice, 3 min). Sections were immersed in 0.1 M triethanolamine (pH 8) for 5 min and incubated in a freshly prepared mixture of 0.25% anhydrous acetic acid in the triethanolamine buffer for 10 min. The slides were washed twice in 2X SSC for 5 min each, dehydrated in 50%,70%, 95% ethanol for 5 min each and air dried under RNAse free conditions.

Hybridization

[α-^{35}S] labeled probe ($5.5x10^8$ cpm/μg, 0.1 ng/μl) was applied to each section in 20 μl of hybridization buffer (750 mM NaCl, 25 mM Pipes pH 6.8, 25 mM EDTA) containing 50% Formamide, 0.2% SDS, 5% Dextran Sulfate, 1X Denhardt's mixture, 100 mM DTT, 250 μg/ml denatured and sonicated salmon sperm DNA and 250 μg/ml polyadenylic acid.

Sections were covered with a siliconed coverslip and incubated for 16-22 h in a humidified chamber at 42°C.

Washes

Coverslips were floated off in 4X SSC and excess of probe was removed by three 15 min washes in 4X SS , one 30 mine wash in 10 mM Tris-HCl (pH 8) containing 20 μg/ml RNAse A (Sigma), 0.5 M NaCl and 1 mM EDTA, two 5 min washes in 2X SSC, and one 30 min wash in 0.01X SSC at 60°C. The sections were dehydrated by passages through 70%, 95% and 100% (V/V) ethanol containing 0.3 M ammonium acetate then air dried.

Detection

The slides were dipped in Ilford K5 emulsion diluted 1/1 with water. After exposure at 4°C for 10 days, the slides were developed in Kodak D-19 and fixed in Hypam Ilford Fix. Sections were stained with hematoxylin, dehydrated and mounted.

Immunofluorescence studies

Chicken organs were removed at embryonic stages according to Hamburger and Hamilton (1951). They were fixed with 4% paraformaldehyde for 2 h. The tissues were mounted in Tissue-Tek OCT medium (Miles Laboratories), rapidly frozen in liquid nitrogen and 10 μm-thick sections were cut at -20°C with cryostat Bright OTF/AS (DIS, D'Hondt Instruments Scientifiques).

Cartilaginous tissue sections were incubated with 5% milk, 0.2% Triton X-100 in PBS for 30 min. Sections were incubated for 1 h at room temperature 1) with monoclonal antibodies against RIHB (Vigny *et al.*, 1989 and unpublished results) or 2) with hybridoma supernatant negative for RIHB or 3) with rabbit antisera against chick type X collagen diluted 1/50, or 4) with a rabbit antisera against chick type II collagen (previously absorbed on DEAE-Trisacryl column) diluted in PBS to 90 μg/ml. Antibodies to collagen type II and X were a kind gift from Dr. Hartman (Institut Pasteur, Lyon). Following three extensive washes with 1% milk in PBS and three washes in PBS, the sections were incubated with fluorescein-isothiocyanate-labeled rabbit anti-mouse IgG or sheep anti-rabbit IgG (Silenus) diluted 1/200 in PBS. After six washes with 1% milk in PBS, they were mounted in 50% glycerol in PBS. After day 6, i.e. stage 28, the cartilaginous sections were pretreated with 1 mg/ml of sheep testis hyaluronidase (type III; Sigma Chemical) in PBS for 30 min at 37°C. Photographs were taken with a Leitz Aristoplan microscope on HP5 film (Ilford).

RNA isolation and Northern blotting

Total RNA was isolated from whole chick wing at different stages (3, 6, 8 and 11 days) by extraction with guanidium thiocyanate and cesium chloride gradient purification (Glifsin *et al.*, 1974). RNAs were separated on an 1% agarose MOPS-formaldehyde gel (10 μg per track) and blotted onto Hybond N nylon membrane (Amersham). Sample quality and quantity were checked by optic density at 260 nm and 280 nm measuring, by ethidium bromide staining and by hybridization of the membrane with probes for chick b actin transcript (not shown).

The membrane was prehybridized at 42°C in a solution containing 50% (w/v) formamide, 5X SSC, 1X Denhardt's, 1% (w/v) SDS, 0.1 mg/ml denatured and sonicated salmon sperm DNA. Hybridization was performed at 42°C for 16 h in the same solution containing chicken RIHB cDNA probe (106 cpm/ml of [α-P^{32}] labeled by random priming). This probe contains the complete RIHB coding sequence and the 3' untranslated region. The final most stringent post-hybridization wash was for 30 min at 65°C in 0.1 XSSC, 0.1% (w/v) SDS. Transcript sizes were estimated by reference to the 18S and 28S ribosomal RNAs and by standard RNA sizes of 0.155 kb to 1.77 kb (BRL).

Quantification of the expression level of RIHB mRNA detected by in situ hybridization

An estimation of silver grain density over the cells in ectoderm or epidermis, presumptive ulna region and ulna at 3, 6, 8 and 11 days was performed using a Biocom 200 Image Analyser (Biocom, Les Ulis, France). The analysis system consisted of a camera linked to a Leitz Aristoplan microscope and monitors. Data were fed directly into a computer system. The ensemble was controlled by a series of Imagenia R. software. After adjusting the image of the monitor, image focusing and image treatment, the computer automatically analyzed the labeling intensity and surfaces on the different structures.

Images were acquired on in *situ* hybridization slides by a Charge Coupled Device (CCD) camera. The slides were examined under oil-immersion light microscopy with transmitted light and a 50x lens. The analogic video images were digitized in the computer in 100 grey levels (expressed from 0 to 255) and converted by segmentation to the number of grains. For a given stage and a studied structure, 15 to 20 fields of 920 μm^2 chosen at random were analyzed on 2 to 4 serial sections. The average number of grains per field was evaluated. The average number of grains was then calculated per cell on the basis of the presence of x cells per field. For each stage, the background was estimated by the grain density over the same surface area of each tissue hybridized with the sense riboprobe. This method of quantification does not provide absolute values of the number of grains, since the power resolution of the analyzer is not sufficient to discriminate between clustered silver grains.

Acknowledgments

This work was supported by grants from MRT, INSERM and AFM. We thank Laurent Jonet, Hervé Coet for expert technical assistance, Edith Jacquemin for her advice in the in situ hybridization experiments and Marie-Paule Hartmann for scanning experiments. We acknowledge Daniel Raulais and Amanda Cockshutt for critical reading of this manuscript. This work would not have been possible without the constant support of Dr. Yves Courtois.

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Accepted for publication: June 1993