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Original Article

HB-GAM/Pleiotrophin: localization of mRNA and protein in the chicken developing leg

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ABSTRACT The heparin-binding growth-associated molecule HB-GAM (also named pleiotrophin) is a developmentally-regulated protein that belongs to a new family of heparin-binding molecules with putative functions during cell growth and differentiation. In order to study the localization of HB-GAM during chicken embryogenesis, we produced specific monoclonal antibodies to this factor. HB-GAM protein is first observed at stage 23 in the developing nervous system and later in the forming cartilage. We present an investigation of the *HB-GAM* mRNA expression and HB-GAM protein distribution in the developing leg by *in situ* hybridization and immunocytochemical studies. We focused our attention on the development of the tibia, where the HB-GAM protein appears at stage 27-28, i.e., just after the condensation of the mesodermal precursor cells of the chondrocytes. The protein then progressively accumulates in the central part of the embryonic cartilage (diaphysis). It persists until stage 42-44 in the regions where hypertrophic cartilage is being replaced by bone marrow. In contrast to the protein, the transcript is first detected at stage 26-27 and later expressed essentially in the epiphysis until stage 37. Therefore the localization of the mRNA does not parallel that of the protein and our data suggest a long half-life of the protein in the hypertrophic cartilage. In addition, the layer of stacked cells surrounding the cartilage core (usually considered as the osteoprogenitor cells) clearly expresses the *HB-GAM* message between stages 30-37 whereas differentiated osteoblasts do not. Furthermore, the distribution of HB-GAM protein in the osteoblast/osteoid layer suggests an involvement of this protein in early steps of osteogenesis. HB-GAM is absent from the newly formed bone.

KEY WORDS: *chondrogenesis, osteogenesis, chicken limb, HB-GAM*

Introduction

The heparin-binding growth-associated molecule, HB-GAM, which was initially purified from rat brain as a neurite outgrowth-promoting protein (Rauvala, 1989), is a developmentally-regulated molecule. This protein was later identified in several laboratories and termed pleiotrophin (Li *et al.*, 1990), heparin affinity regulatory peptide (HARP) (Courty *et al.*, 1991) or heparin-binding neurite-promoting factor (HBNF) (Böhlen *et al.*, 1991). Interestingly, using the differential hybridization screening method, a cDNA clone coding for HB-GAM was isolated from a murine osteoblastic cell line MC3T3 and termed OSF1 for Osteoblast Specific Factor-1 (Tezuka *et al.*, 1990). The *HB-GAM* cDNA encodes a lysine rich, highly basic protein of 168 amino acids with a 32 -amino acid signal sequence that is highly conserved in the human, rat, bovine and chicken proteins (Hampton *et al.*, 1992). HB-GAM presents 50% identity with the amino acid sequence of the mammalian MK or midkine protein (for midgestation kidney) (Tomomura *et al.*, 1990) and of the chicken RIHB (Retinoic acid-Induced Heparin-Binding) protein (Vigny *et al.*, 1989; Urios *et al.*, 1991). The developmental distribution (Kadomatsu *et al.*, 1990; Duprez *et al.*, 1993; Muramatsu *et al.*, 1993; Cockshutt *et al.*, 1994), the regulation by retinoic acid in some cell types in culture (Huang *et al.*, 1990; Raulais *et al.*, 1991; Cockshutt *et al.*, 1993) and the overall structure of the genes (Matsubara *et al.*, 1990; Duprez *et al.*, 1994) strongly suggest that RIHB is the chicken counterpart of MK. *MK/RIHB* mRNA and MK/RIHB protein are highly expressed during early embryogenesis by all three germ layers (Kadomatsu *et al.*, 1990; Cockshutt *et al.*, 1994). In contrast, Northern Blot analysis performed by different groups indicates that *HB-GAM* mRNA is expressed during late mammalian development, for example during the perinatal period of the rat development (Rauvala, 1989). In addition, this expression is restricted to the developing central nervous system, the muscle and possibly the cartilage (Li *et al.*, 1990; Merenmies and Rauvala., 1990; Tezuka et *al.*, 1990; Mitsiadis et al., 1995; Nolo *et al.*, 1996).

The exact biological activities of HB-GAM, particularly *in vivo*, remain largely unknown. *In vitro*, HB-GAM has been shown to promote neurite outgrowth of rat embryonic neurons in primary cultures (Rauvala, 1989). In addition, the localization of HB-GAM during the development of the rat central nervous system (Wanaka

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et al., 1993; Nolo *et al.*, 1995) strongly suggests that HB-GAM supports the axonal growth of central neurons during development. The neurotrophic effect of HB-GAM is largely accepted. It is also a protooncogene; HB-GAM is highly expressed in many human tumors specimens (Wellstein *et al.*, 1992) and cells transformed by the *HB-GAM* gene form highly angiogenic tumors when implanted into the nude mice (Zhang *et al.*, 1997). A mitogenic activity has been reported by different laboratories (Fang *et al.*, 1992; Laaroubi *et al.*, 1994; Souttou *et al.*, 1997). A recent report, however, indicates that exogenous HB-GAM strongly inhibits proliferation of rat embryonic mesenchymal cells (Szabat and Rauvala, 1996). It has been also reported that HB-GAM can increase the plasminogen activator activity of bovine aortic endothelial cells in culture (Kojima *et al.*, 1995). This activity could explain some of the modulatory actions of HB-GAM in differentiation and morphogenesis of the vertebrate embryo. Finally, HB-GAM has been suggested to play a fundamental role in cartilage and/or bone formation (Tezuka *et al.*, 1990). The presence of HB-GAM in the rib cartilage of the 14.5 day old mouse embryo has been reported (Mitsiadis *et al.*, 1995) but analysis of HB-GAM expression and localization during embryonic development of the cartilage and bone of the vertebrate limb has not yet been described. This analysis would be an essential prerequisite for understanding the role of this protein in these processes.

We therefore produced specific monoclonal antibodies (Mabs) against chicken HB-GAM in order to study the localization of this protein during chicken embryogenesis. The distribution of the *HB-GAM* transcript was analyzed in parallel by *in situ* hybridization. The presence of HB-GAM is essentially restricted at first to the central nervous system (CNS) at stage 23-24 and later to the developing cartilage. In this paper, we focused our attention on the expression and localization of both message and protein in the developing tibia. The spatial and temporal patterns of distribution of the message and of the protein suggest that HB-GAM is an essential factor of both chondrogenesis and early steps of osteogenesis.

Results

Studies of the expression of HB-GAM first required the preparation of pure protein and specific anti-HB-GAM antibodies.

Purification of chicken HB-GAM and antibody production and characterization

Chicken HB-GAM was purified from brains of 15-18 day old embryos (see experimental procedures). HB-GAM eluted from the Mono S column reproducibly gave two bands after SDS-PAGE which were only recognized by anti-HB-GAM antibodies (see below) in a Western Blot analysis (Fig. 1). One band might correspond to a proteolytic breakdown product, as previously shown by different laboratories (Böhlen *et al.*, 1991; Souttou *et al.*, 1997). However, the existence of an extended form of HB-GAM containing three extra amino acids at the N-terminal end has been reported (Laaroubi *et al.*, 1994). Thus the two bands could correspond to the two forms differing in their NH2 terminal sequence.

Immunization of rabbits with chicken HB-GAM yielded sera with unclear specificities and ELISA titres at least ten times lower than those previously obtained using RIHB as the antigen (Vigny *et al.*, 1989; Cockshutt *et al.*, 1994). In particular, we could not completely exclude a weak cross reactivity with RIHB, as tested by ELISA. This fact is not completely surprising for two reasons. First, MK/RIHB and HB-GAM share 50% identity in terms of amino acid sequences. Second, we have previously obtained a Mab which reacted with both chicken HB-GAM and RIHB (Beau *et al.*, 1995). In addition, the use of polyclonal antibodies against HB-GAM (in order to secure their specificity) usually required affinity-purified antibodies (Mitsiadis *et al.*, 1995; Nolo *et al.*, 1995; Szabat and Rauvala, 1996). Taking account of all these data, we chose to produce monoclonal antibodies to chicken HB-GAM.

Two Mabs, 5A12 and 6D2, were obtained. They were specific for HB-GAM and did not recognize RIHB or human or mouse MK as tested by ELISA and Western blots. In addition, these Mabs recognized chicken, human and rat HB-GAM, showing that the corresponding epitopes are conserved between species. Both antibodies react with chicken HB-GAM after Western blotting (Fig. 1). The epitopes recognized by the two Mabs are distinct because the binding of 5A12 to HB-GAM was not affected by the presence of heparin whereas heparin (10 μg/ml) abolished the binding of 6D2 (data not shown).

We used essentially the 5A12 Mab for the immunocytochemical analysis of the HB-GAM localization. Identical results, however, were obtained using affinity purified antibodies to rat HB-GAM (a kind gift of Dr Rauvala). It is noteworthy that both polyclonal and monoclonal antibodies recognize the same two bands after Western blotting (Fig. 1). The localization of RIHB was revealed using the previously described anti-RIHB Mab (named 4C7, Cockshutt *et al.*, 1994), which does not recognize HB-GAM as tested by ELISA and Western blots (Fig. 1). Control experiments were performed using a control Mab which recognizes neither HB-GAM nor RIHB and which revealed no staining at any developmental stage examined.

Distribution of HB-GAM mRNA and protein in the developing limb

The chondrocyte differentiation program follows a series of successive steps during the development of the embryonic limb (see for reviews Pechak *et al.*, 1986a,b; Cancedda *et al.*, 1995). First, mesodermal precursor cells condense and differentiate into chondrocytes expressing specific markers such as type II collagen. Then, chondrocytes progress through a differentiation program, starting as proliferating chondrocytes in the epiphyseal growth plate and ending up as hypertrophic chondrocytes in the diaphyseal region. The differentiation of chondrocytes into hypertrophic chondrocytes is characterized by the production of type X collagen (Schmid and Linsenmayer, 1985; Duprez *et al.*, 1993). It is generally thought that hypertrophic chondrocytes in the cartilage core do not contribute to initial bone formation in the developing tibia. Their ultimate fate is death and hypertrophic cartilage is later replaced by bone marrow (Pechak *et al.*, 1986b). In this model, osteoblasts originate from a layer of stacked cells peripheral to the cartilage core (see below) (Pechak *et al.*, 1986a). Other investigators, however, have suggested that some hypertrophic chondrocytes are transformed, at least *in vitro*, into osteogenic cells (Cancedda *et al.*, 1995; Roach *et al.*, 1995 and references therein).

We examined the expression and distribution of HB-GAM in the developing chick limb, focusing our studies essentially on the tibia. HB-GAM protein was detected by immunoperoxidase staining, the sections being counterstained with hemalun blue solution. *HB-*

GAM mRNA expression was visualized by *in situ* hybridization using a specific chicken probe (a kind gift of Dr Rauvala, see experimental procedures).

Stages 20-24

The pattern of HB-GAM immunostaining is shown for sections cut from stage 20 and 24 chick embryos in Figure 2. Transverse sections of the embryos were made at the level of the leg. At stage 20-22 (day 3) HB-GAM was not expressed (Fig. 2A). At the same stage the localization of RIHB protein was almost uniform in the limb (Fig. 2B) with the noticeable exception of the apical ectodermal ridge (AER) displaying a non uniform staining (Fig. 2C). At stage 24 (day 4) the most intense immune reaction for HB-GAM was found in a particular region of the neural tube corresponding to the bundles of His, where primary sensory neurons project (Fig. 2D). This localization is identical to that recently reported by Nolo and collaborators (Nolo *et al.*, 1996) using purified antibodies to rat HB-GAM. At the same stage, in the limb, the zone of mesenchymal cell condensations (as revealed with the toluidine blue staining) that correspond to the first step in the formation of the cartilage did not contain any *HB-GAM* transcript or protein HB-GAM immunoreactivity (not shown).

Stages 26 to 28 (day 5.5 to day 6)

The distribution of the HB-GAM protein and the expression of its mRNA in the 5.5 day old embryonic leg is shown in Figure 2E and F. At that stage the transcript was detected in the femur but was hardly discernible in the cell condensation corresponding to the future tibia (Fig. 2F). These data reflect the fact that the chondrocyte differentiation program is more advanced in the femur than in the tibia. The distribution of the protein in the femur did not strictly parallel that of the message (Fig. 2E). The transcript was uniformly expressed by all the chondrocytes whereas the protein was more concentrated on the edges of the forming cartilage i.e., in the collar cell layer surrounding the cartilage core and in the loose mesenchyme peripheral to this cell layer (see Pechak *et al.*, 1986a; Rooney and Archer, 1992 for a complete description). It is also noteworthy that the embryonic muscle, detected with the specific antibody 13F4 (Rong *et al.*, 1987), was devoid of staining (Fig. 2G). At stage 28, both protein and message were now clearly visualized in the tibia. Their distributions were identical to those described at stage 26-27 for the femur (not shown). At these stages (26 to 28), RIHB was still largely distributed in the limb bud (see Duprez *et al.*, 1993).

Stages 31-32 (day 7.5)

The immunocytochemical signal observed in the cartilage of the 7.5 day old chick tibia is worthy of comment. In the future epiphyseal region the staining was very faint but clearly detectable at higher magnification (not shown). From the epiphysis to the central diaphysis, there was clearly a gradient in the distribution of the HB-GAM protein. It was concentrated in the region of the future diaphysis occupied by early hypertrophic chondrocytes (Fig. 3A). At higher magnification the staining appeared to be concentrated at the surface of the hypertrophic chondrocytes whereas the matrix in this region was much less stained (Fig. 3C).

At this stage the structure of the osteogenic collar surrounding the cartilage core at the mid-diaphysis is complex. According to

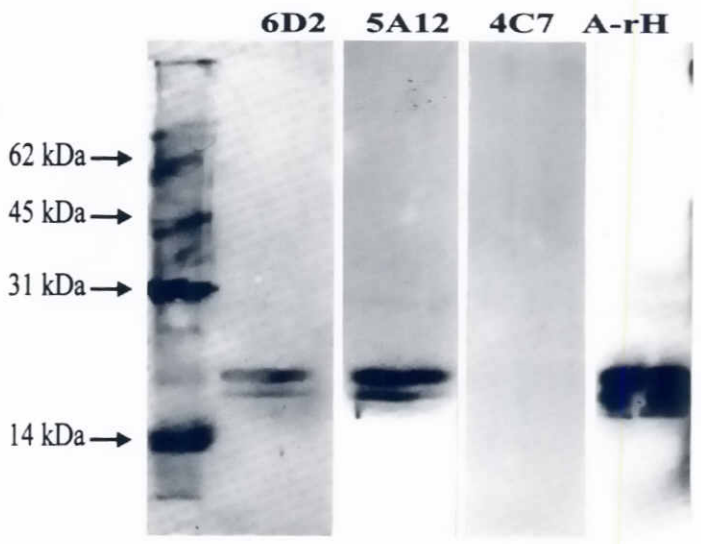

Fig 1: Western blots of chicken purified HB-GAM with Mabs 6D2 and 5A12 specific for HB-GAM, with a Mab 4C7 specific for RIHB and with rabbit affinity purified polyclonal antibodies to rat HB-GAM (A-rH) (a gift of Dr. Rauvala). Biotinylated molecular weight markers were obtained from Biorad.

Pechak and collaborators (Pechak *et al.*, 1986a), from the central core of hypertrophic cartilage to the periphery, one can distinguish several layers of cells and acellular zones (see Fig. 3C and D). A distinct layer of elongated peripheral chondrocytes is present at the outer aspect of the cartilage core. In contrast to the hypertrophic chondrocytes present in the central core, this layer of flat or elongated chondrocytes was devoid of staining (Fig. 3C). Continuing radially, the next layer or collar is composed of round osteoblastic cells which is separated from the layer of elongated peripheral chondrocytes by a definite acellular zone often referred to as osteoid. Osteoid corresponds to the premineral zone but at this stage mineralization has not yet occurred. An intense staining was observed in the osteoid and around the osteoblasts (Fig. 3C). Peripheral to the osteoblast collar is the layer of stacked cells which could form a stratum of osteoprogenitor cells as proposed by Pechak *et al.* (1986a,b). Considering the HB-GAM immunoreactivity, a very weak staining was detected in this cell layer.

The localization of the *HB-GAM* transcript in the forming cartilage is shown in Figure 3B and D. The mRNA was observed primarily in the epiphyseal region (Fig. 3B), but the expression was not uniform. At the epiphyseal head some overlapping cells could be seen separating the chondrocytes from mesenchyme (Rooney and Archer, 1992). These cells clearly expressed the transcript. Underneath, the *HB-GAM* mRNA was weakly detectable in an area occupied by round polygonal chondrocytes surrounded by small amounts of extracellular matrix. This region presented a compact structure and could correspond to the reserve zone of resting cells. Beneath, the round chondrocytes of the proliferative zone (mitoses were evident within some cells) strongly expressed the message and this expression extended to the first layers of the flattened chondrocytes. Then, *HB-GAM* expression declined rapidly approaching the hypertrophic zone (Fig. 3B). Thus, the distribution of the mRNA did not parallel that

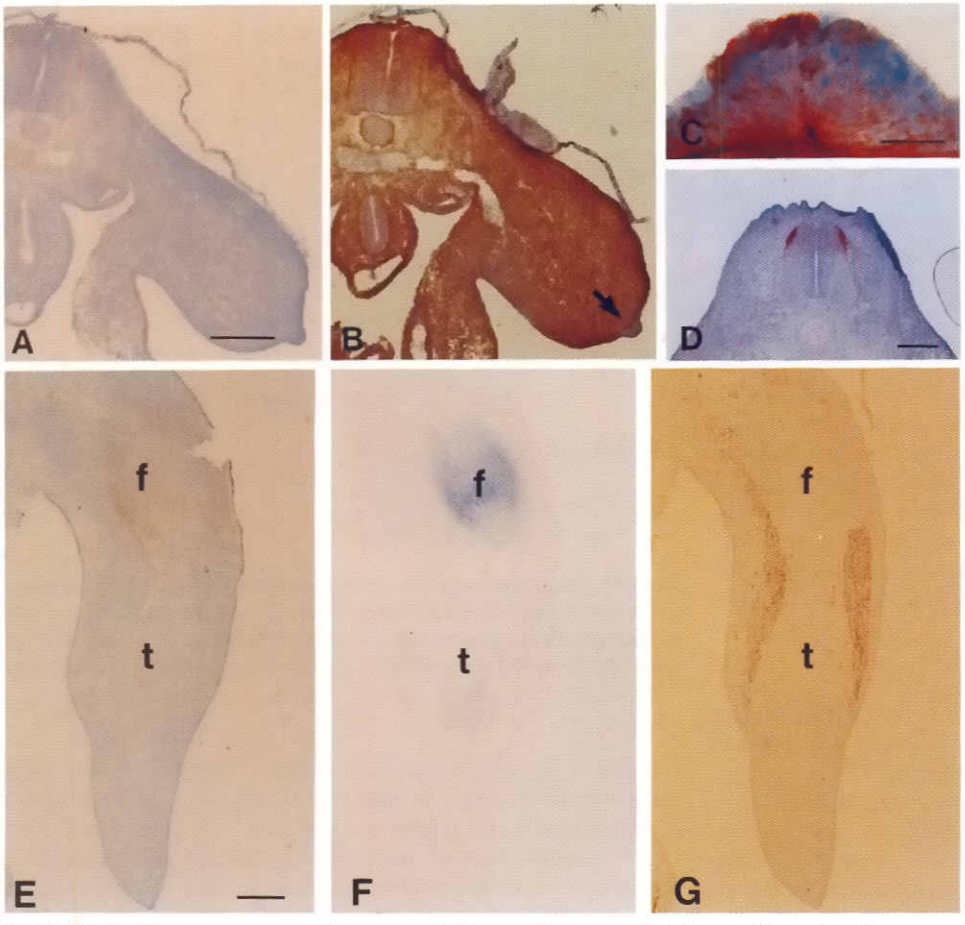

Fig. 2. HB-GAM appearance during early chicken embryogenesis (stages 22 to 27). *Transverse sections of stage 22 embryos were stained with the anti-HB-GAM Mab 5A12* **(A)** *or with the anti-RIHB Mab 4C7* **(B)** *(Bar, 250 μm).* **(C)** *Higher magnification of the AER region indicated by the arrow in B (Bar, 25 μm). Localization of HB-GAM immunoreactivity in the spinal cord* **(D)** *at stage 24 (Bar, 250 μm). At this stage no message or protein was visualized in the limb bud (not shown). Longitudinal sections of the proximal part of the leg at stage 27 stained with the anti-HB-GAM Mab 5A12* **(E)** *or with the Mab 13F4 which specifically recognises the post-mitotic muscles cells* **(G)**. *HB-GAM immunoreactivity was hardly discernible in the tibia (t). The distribution of HB-GAM in the femur (f) was not uniform. The staining of HB-GAM in the zone of the collar cell layer surrounded the cartilage core (Bar, 250 μm).* **(F)** *Expression of the HB-GAM message revealed by in situ hybridization in the limb bud at the same age (stage 27). Note, in contrast to the protein, that the transcript was uniformly distributed in the forming femur. f, femur; t, tibia.*

of the protein as previously shown for RIHB (Duprez *et al.*, 1993). This difference of distribution suggests that HB-GAM is synthesized and secreted by the proliferative chondrocytes of the future epiphysis and then accumulates in the matrix and around the cells of the maturating and hypertrophic zones. The other striking observation was the presence of the *HB-GAM* transcript in the stacked cell layer (Fig. 3B and D). The message was present in this cell layer all along the cartilage rudiment and therefore extended well beyond the hypertrophic cell region (Fig. 3B). At this stage, the stacked cell zone is 8-10 cell layers in diameter and it is noteworthy that the message was expressed in the more external of these cell layers (Fig. 3D). Assuming that the stacked cells correspond to the osteoprogenitor cells, these data suggest that the expression of *HB-GAM* mRNA is concomitant to and may be an essential step of the early phase of osteoblast differentiation.

Stage 34 (day 8-9)

The cellular arrangement at stage 34 is the same as in the stage 31 material. However, the length of the portion of the cartilage rudiment that contains hypertrophic chondrocytes has increased along with the length of the osteoblast collar. The mid-diaphysis is now surrounded by the mineral ring. Distal to the mineralizing front, there is the acellular premineral zone or osteoid, as seen at the mid-diaphysis itself at earlier stages. In other words, the osteoid and osteoblast layers spread from the center towards the ends of the rudiment ahead of the mineral collar.

The localization of the message is roughly equivalent to that described at stage 31-32 (not shown). The pattern of expression of the HB-GAM protein in the hypertrophic zone of the tibia revealed interesting features, some of them different of that observed at stage 31-32 (Fig. 3E,F and G). The distributions of the HB-GAM protein at the mid-diaphysis and in the regions located on both sides of the mid-diaphysis were not identical. In the mid-diaphysis, all the hypertrophic chondrocytes were stained with the HB-GAM antibody which was not the case at stage 32. Some cells were intensively stained whereas in others the HB-GAM immunoreactivity was much weaker (see also Fig. 3F). The cartilage matrix of this region clearly contained HB-GAM. In addition, the mineral and osteoblast layers surrounding the cartilage were devoid of staining (Fig. 3F). In contrast, in the regions located on both sides of the mid-diaphysis only the cartilage matrix was stained but the staining was more intense in the interior of the cartilage core than in the peripheral zone (Fig. 3E and G). Furthermore, the interface of the cartilage and osteoblast layer was intensely stained (Fig. 3G, bold arrow). Finally, the osteoblast layer itself clearly contained HB-GAM which was localized extracellularly. The pattern of expression in the region located on both sides of the mid-diaphysis at this stage is similar to that previously found at the mid-diaphysis itself at stage 32.

At this stage, type II collagen, a marker of the cartilage matrix, is uniformly distributed in the entire length of the cartilage, while type X collagen, a marker of hypertrophic chondrocytes, only begins to be weakly expressed in the cartilage matrix of the mid-diaphysis (Schmid and Linsenmayer, 1985; Duprez *et al.*, 1993). Note that the embryonic muscle and epidermis were devoid of HB-GAM.

Stage 37 (day 11)

The localization of HB-GAM in the cartilage matrix from the diaphysis to the epiphysis is illustrated, Figure 4A. When compared

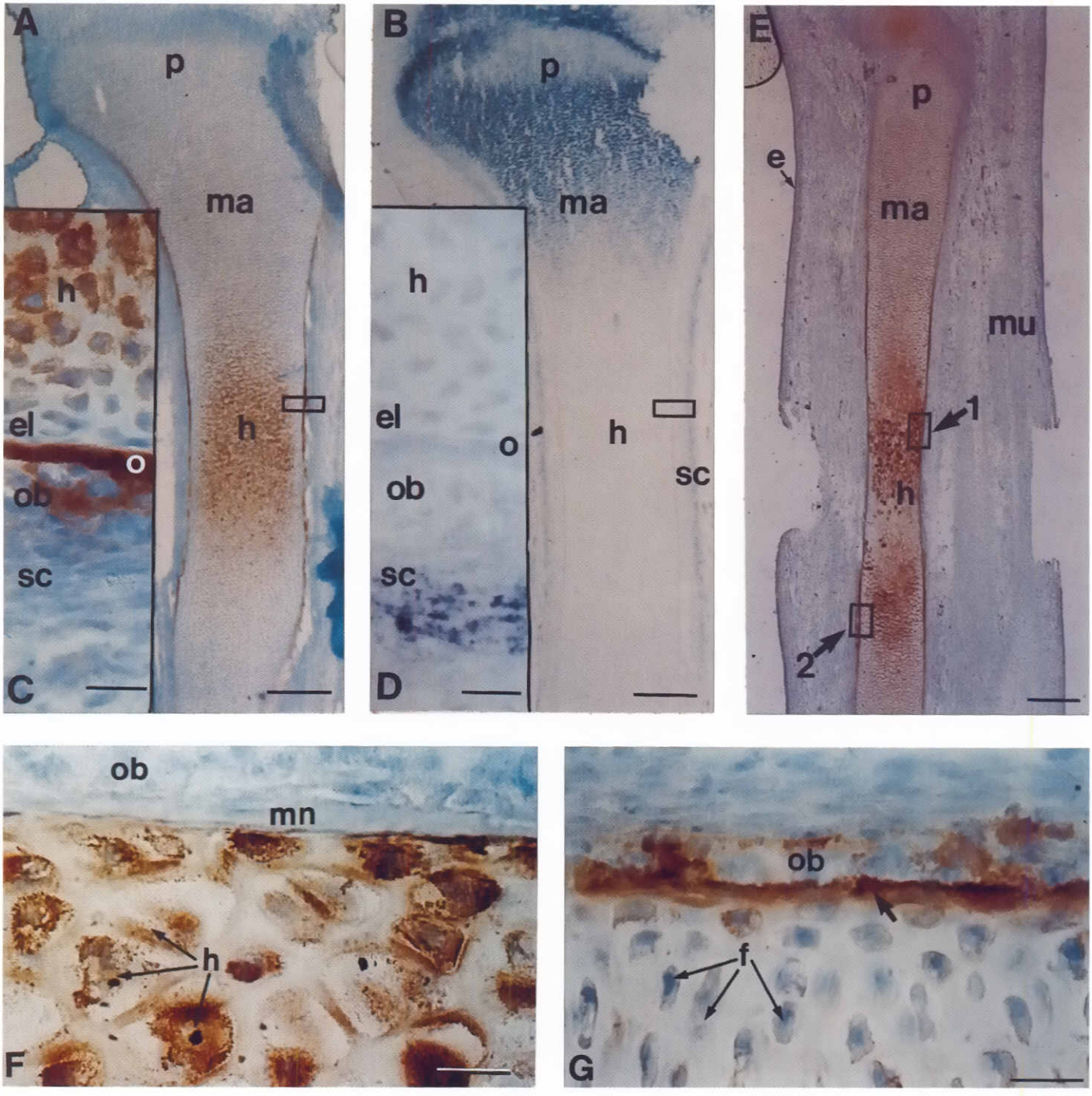

Fig. 3. HB-GAM expression in the embryonic leg at stages 31-32 and 34. *Longitudinal section of the leg at stage 31-32 stained with the anti-HB-GAM Mab 5A12* **(A)** *(Bar, 250 μm).* **(C)** *Higher magnification of the mid-diaphysis region indicated by the rectangle in A (Bar, 25 μm).* **(B)** *HB-GAM mRNA expression revealed by in situ hybridization on a section adjacent to the section shown in Figure 3A. Note the presence of the transcript essentially in the epiphyseal region and in the stacked cell layer.* **(D)** *Higher magnification of the mid-diaphysis region indicated by a rectangle in B (Bar, 25 μm).* **(E)** *Longitudinal sections of tibia at stage 34 stained with the anti-HB-GAM Mab 5A12 (Bar, 250 μm).* **(F and G)** *higher magnification of two regions of (E) indicated by the arrows; arrow 1=(F), arrow 2=(G) (Bars, 20 μm). In (G) the bold arrow shows the staining of HB-GAM at the interface of the cartilage and osteoblast layer. e, epidermis; el, diaphyseal elongated peripheral chondrocytes; f, flattened chondrocytes of the zone of maturation; h, hypertrophic diaphyseal chondrocytes; ma, zone of maturation; mn, mineral layer; mu, muscle; o, osteoid; ob, osteoblast layer; p, proliferative chondrocytes; sc, stacked cell layer.*

to stage 34 several major differences were observed. First, the epiphyseal region was devoid of HB-GAM immunoreactivity. The second difference concerns the distribution of HB-GAM in the mid-diaphysis. The initial stages of the formation of the marrow cavity occur at stage 35 (Pechak *et al.*, 1986b). At stage 37 the resorption of uncalcified cartilage can be visualized by the presence of large

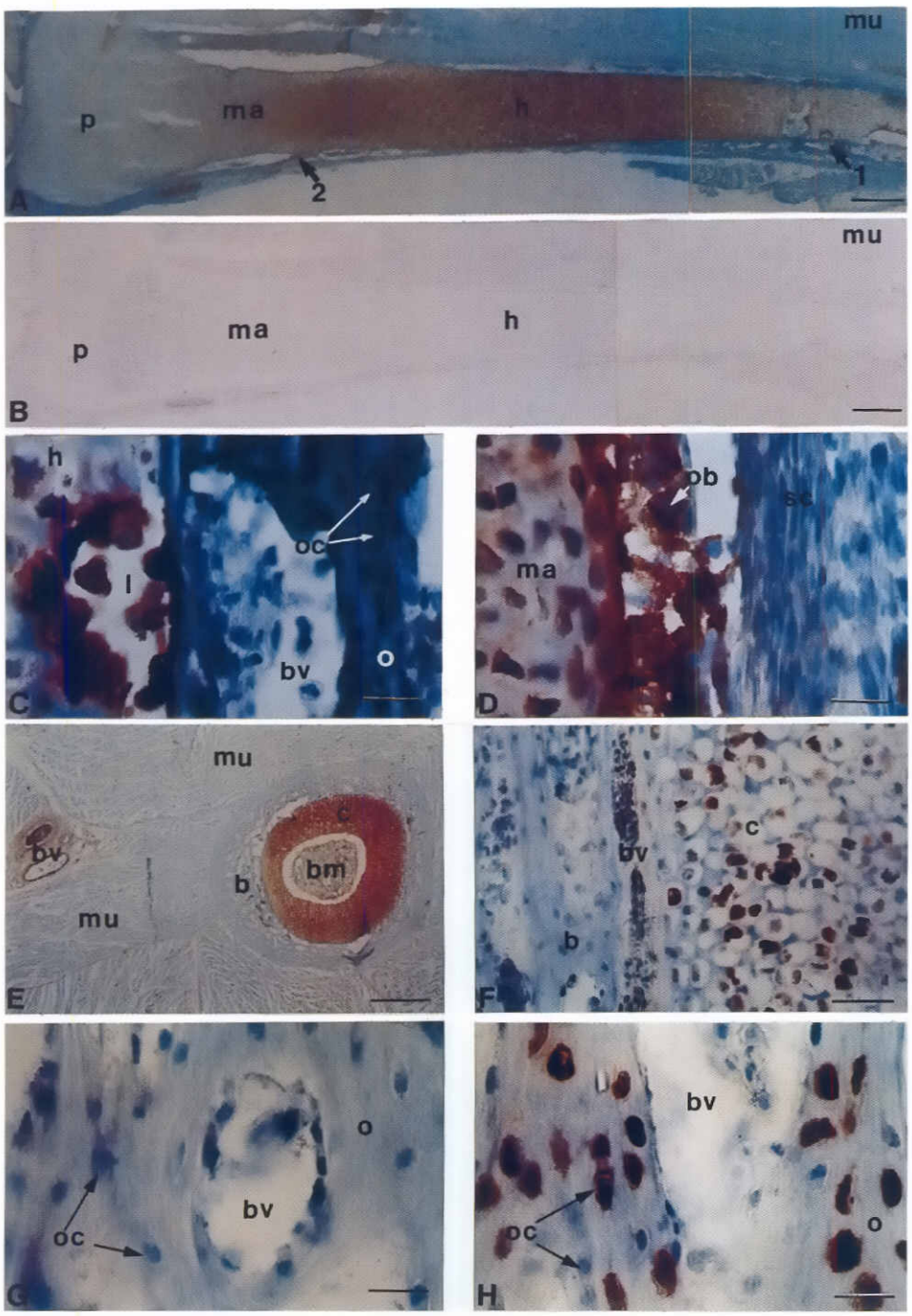

Fig. 4. HB-GAM expression in the embryonic leg at stages 37 (day 11), 39 (day 13) and 44 (day 18). *Longitudinal sections of the tibia at stage 37 stained with the anti-HB-GAM Mab 5A12.* **(A)** *(Bar, 250 μm).* **(C and D)** *Higher magnification of two regions of (A) indicated by the arrows; arrow 1=(C), arrow 2=(D) (Bars, 20 μm).* **(B)** *HB-GAM mRNA expression revealed by in situ hybridization on a section adjacent to the section shown in Figure 4A (Bar, 250 μm). The presence of the message is hardly discernible (see for comparison Fig 3B).* **(E)** *Transverse section of a 13 day old leg at the level of the hypertrophic zone of the tibia, distal to the mid-diaphysis, stained with the anti-HB-GAM Mab 5A12 (Bar, 500 μm).* **(F)** *Transverse section at day 18 in the hypertrophic cartilage/bone area stained with the anti-HB-GAM Mab 5A12 (Bar, 50 μm). Sections of embryonic bone at day 18 stained with the anti-HB-GAM Mab 5A12* **(G)** *or with the anti-RIHB Mab 4C7* **(H)** *(Bars, 20 μm). b, bone; bm, bone marrow; bv, blood vessels; c, cartilage; h, hypertrophic diaphyseal chondrocytes; l, lacunae; ma, zone of maturation; mu, muscle; ob, osteoblast layer; oc, osteocytes; p, proliferative chondrocytes; sc, stacked cell layer.*

cavities. The surface of these lacunae was highly immunoreactive (Fig. 4C) but the staining in the matrix of the mid-diaphysis itself was much fainter (Fig. 4A and C) than in the adjacent regions. This phenomenon was hardly discernible at stage 34 (Fig. 3E). This result suggests that the progressive replacement of the cartilage by bone marrow in the mid-diaphysis is accompanied or preceded by a decrease of HB-GAM in the matrix of the hypertrophic cartilage. It is also noteworthy that in the hypertrophic zone distal to the mid-diaphysis some cells were intensively stained with the HB-GAM antibody (Fig. 4A). At stage 37, the collar of mineral has largely spread from the diaphysis to the ends of the rudiments. This ring and surrounding osteocytes were devoid of staining. Distally there are the regions where mineralization has not yet occurred. In this region an intense immunoreactivity was visualized around the osteoblasts and in the osteoid/flat chondrocyte border (Fig. 4D).

The expression and distribution of the *HB-GAM* transcript is shown in Figure 4B. Firstly no *HB-GAM* mRNA was detected in the stacked cell layer and/or the osteogenic collar. Secondly only a very weak expression, when compared to stage 32-34, could be visualized in the epiphyseal regions. The presence of the protein in the cartilage matrix of the hypertrophic zone in the almost total absence of transcript suggests that the protein has a long half-life.

Stages 39 to 44 (day 13 to day 18)

At these stages no transcript was visualized. Transverse sections of the tibia, distal to the mid-diaphysis, at stage 39 (13 day) (Fig. 4E) show that HB-GAM is still present in the cartilage matrix of this region. Then HB-GAM progressively disappeared from the matrix where no staining could be detected after stage 41 (day 16). However, in the region where hypertrophic cartilage will be replaced by vascular and marrow elements (Pechak *et al.*, 1986), some cells were always intensively stained until stage 42-44 (day 17-18) (see Fig. 4F) In contrast, the newly formed bone was completely devoid of staining, showing that osteocytes do not express HB-GAM (Fig. 4G). At this stage, it is also noteworthy that in the newly formed bone the

presence of RIHB could be detected in some but not all osteocytes (Fig. 4H).

Discussion

This paper focuses on the HB-GAM expression during chondrogenesis and osteogenesis in the tibia. The expression of the transcript was analysed by *in situ* hybridization and the distribution of this protein was revealed using specific Mabs against HB-GAM. The spatial and temporal patterns of distribution of the message and of the protein suggest that HB-GAM is an essential factor of both chondrogenesis and early steps of osteogenesis.

Chondrogenesis

HB-GAM did not appear to be involved in the mesenchymal cell condensation, as the transcript and the protein were detected just after this process (at stage 26-28). Thus, the early phase of chondrocyte differentiation was concomitant with the appearance of HB-GAM.

Between stages 31-37, the differences between the expression of the mRNA and the distribution of the protein recalls those previously found for RIHB (Duprez *et al.*, 1993). Such differences, to a lesser extend, have also been recently reported in the chicken neural tube at stage 24 (Nolo *et al.*, 1996). To explain our results, we have to consider two facts. First HB-GAM is a secreted protein with a classical-type secretion signal. It is for example essentially found in the culture medium of different cell systems *in vitro*. Second the increase in length of embryonic cartilages corresponding to long bones, such as the tibia, during embryonic development is considerable. At stage 31-32, the message was expressed in the epiphysis whereas the protein was concentrated in the central part of the forming tibia. Therefore, HB-GAM was synthesized by the proliferative and/or post-proliferative chondrocytes of the future epiphysis and then accumulated in the hypertrophic zone. This result first suggests that HB-GAM receptors or binding molecules were mainly present in the matrix or at the surface of the chondrocytes located in the flattened and hypertrophic zones. Second, the progressive concentration of HB-GAM in the future diaphyseal region just at the onset of the hypertrophic process i.e., before the appearance of type X collagen, suggests that this protein could be involved in this differentiation process.

At stage 34, some hypertrophic chondrocytes in the mid-diaphyseal region displayed a strong HB-GAM immunoreactivity. The fact that staining of hypertrophic chondrocytes varied in intensity from cell to cell, suggests that this cell population is heterogeneous. One could propose that the hypertrophic process involves different steps, one being characterized by an intense accumulation of HB-GAM. The presence of HB-GAM has also been reported in the adult bovine nasal cartilage (Neame *et al.*, 1993). It is noteworthy that in this case the protein was not present in the matrix but located either intracellularly or at the cell surface (as in some avian hypertrophic chondrocytes).

Finally, at stage 37, the message has virtually disappeared. Therefore, the presence of the protein in the cartilage matrix of the hypertrophic zone distal to the mid-diaphysis, in absence of any detectable transcript, suggests a long half-life of the protein. In addition, the staining in the mid-diaphysis itself was greatly reduced. At this stage, blood vessels enter the hypertrophic cartilage at the mid-diaphysis. The dissolution of the first layer of bone by marrow elements has begun and the hypertrophic cartilage is progressively replaced by bone marrow. The process of cartilage matrix resorption is therefore accompanied by a progressive disappearance of HB-GAM. At this level, HB-GAM could also act as an angiogenic factor since its angiogenic properties have already been demonstrated (Laaroubi *et al.*, 1994).

Based on the expression of CMP (Cartilage Matrix Protein), Chen et collaborators (Chen *et al.*, 1995) have proposed that between the proliferative and the hypertrophic zones there exists a region called the maturation zone. This region contains postproliferative chondrocytes which do not express type X collagen. These observations are consistent with a model which proposes that the chondrocyte differentiation program could be divided into at least three distinct stages: proliferation, maturation and hypertrophy (Chen *et al*, 1995). The *CMP* message is essentially expressed in the maturation zone but the protein is present both in the maturation zone and in the hypertrophic zone. The HB-GAM protein distribution is therefore reminiscent of that found for CMP. The analysis of the CMP distribution, however, has been performed between stages 40 and 45 in the tibiotarsus, i.e., much later than the present work. A direct comparison at different stages of the expression of HB-GAM, and CMP would probably help to understand the different or additional roles of these proteins during the maturation and hypertrophic processes.

Osteogenesis

The expression of both mRNA and protein in the osteogenic collar between stage 31 and 37 is worthy of comments. At stage 31-32, the stacked cells, usually considered as the osteoprogenitor cells, clearly expressed the transcript. The message was present in this cell layer all along the cartilage rudiment and therefore extends beyond the hypertrophic cell region. In addition, the message was essentially expressed in the more external stacked cells. At stage 37, no transcript was visualized in the osteogenic collar. Thus *HB-GAM* mRNA was expressed only in the early phase of osteoblast differentiation process. In this context, it is noteworthy that a cDNA clone coding for HB-GAM has been previously isolated from the murine osteoblastic cell line MC3T3 and termed OSF1 for Osteoblast Specific Factor-1 (Tezuka *et al.*, 1990). This data and our results strengthen the hypothesis that stacked cells are actually osteoprogenitor cells at least in the chicken perichondral bone formation.

Considering the protein, its distribution was different from that of the message. HB-GAM was weakly present in the stacked cells layer which expresses the mRNA. HB-GAM, however, was essentially detected at the surface of the round osteoblasts and at the border between the osteoid/osteoblast layer and the elongated peripheral chondrocyte layer. N-Syndecan/Syndecan 3, a heparan sulfate proteoglycan identified as a neuronal receptor for HB-GAM (Raulo *et al.*, 1994), has been reported to be present along the mid-diaphysis of the proximal skeletal elements of the stage 31 wing (Gould *et al.*, 1995). This region corresponds to an area in which osteoblasts are differentiating. It is therefore tempting to suggest that HB-GAM secreted by the stacked cells binds to the Syndecan 3 present on the surface of the osteoblasts. A direct comparison of the distribution of both Syndecan 3 and HB-GAM during the development (i.e., at different stages) of the tibia would probably be informative. At stage 31-32 the osteoblasts/osteoid/flat peripheral chondrocyte region was stained with HB-GAM antibody all along the cartilage rudiment.

Then this staining disappeared in the region where the mineralization has occurred. This mineralization process starts at stage 33-34 and the mineral ring progressively expands from the diaphysis to the epiphysis. This ring and surrounding osteocytes were devoid of staining. In contrast, in the region where the cartilage rudiment was still surrounded only by the osteoid and osteoblast layers an intense immunoreactivity was visualized. This result is again in favor of an involvement of HB-GAM in the early steps of osteoblasts differentiation and bone formation.

HB-GAM distributions in mammals and birds embryonic tissues

HB-GAM has been observed in newly formed bone in the mouse (Mitsiadis *et al.*, 1995) whereas the chicken bone was completely devoid of HB-GAM immunoreactivity. However, the processes of chondrogenesis and initial bone formation in avian and mammalian embryos have major differences (Cancedda *et al.*, 1995 and references therein). These differences could explain the variability in the HB-GAM expression in the newly-formed bone between mammals and birds. We noticed another difference between mammalian and avian embryonic development. The distributions of rat (Szabat and Rauvala, 1996) and chicken HB-GAM (this work) in the embryonic muscle do not appear similar. Our data show that, between stages 22 to 39, avian HB-GAM was expressed only in the cartilage. In comparison, rat HB-GAM was detected very early (embryonic day 12, roughly equivalent to stage 18-19 of the avian development) in the embryonic limb mesenchyme. It was uniformly distributed in the differentiated muscle at day 15 (roughly equivalent to stage 28-30 of the avian development) and appeared as patches at the new formed neuromuscular junctions at day 17 (roughly equivalent to stage 35-37 of the avian development; Szabat and Rauvala, 1996). The antibodies used in the two studies are not identical, but we first detected HB-GAM, at stage 24, in a particular region of the neural tube (see Fig. 2D) and this localization is identical to that recently reported by Nolo and collaborators (Nolo *et al.*, 1996) using purified polyclonal antibodies to rat HB-GAM i.e., the same antibodies used by Szabat and Rauvala (1996). Taking account of the differences between the time-courses of rat or mouse and chicken embryonic developments, these results could suggest a variability in the tissue localization of the HB-GAM protein between species.

The HB-GAM expression in the developing nervous system is well documented. Our findings emphasizes the strong HB-GAM expression during cartilage and bone development, at least in the chick. Further studies should address how HB-GAM plays key roles in chondrogenesis and osteogenesis.

Materials and Methods

Materials

Fertilized eggs of white Leghorn chickens were obtained from Hass (Kaltenhouse, France). Male Balb/c mice were purchased from Iffa Credo (France). Heparin-Sepharose and the Mono S column were obtained from Pharmacia (Sweden). The mounting material, Tissue Tek OCT compound, was obtained from Miles (Bayer Diagnostics, Domont, France). When not specified in the text the chemicals were from Sigma.

Purification of Chicken HB-GAM and specificity of antibadies

Chicken HB-GAM was purified from 15-18 day embryonic chick brains, essentially as previously described (Beau *et al.*, 1995). The fraction eluted from the heparin-Sepharose column at 1M NaCl was analysed by ELISA (Rennard *et al.*, 1980) using specific Mabs to HB-GAM and to RIHB. This fraction clearly contained RIHB. RIHB contamination was removed on an anti-RIHB monoclonal immuno affinity column (Vigny *et al.*, 1989). HB-GAM from the unretained material was finally purified on a Mono S column with a NaCl gradient (Milner *et al.*, 1989).

Aliquots of rat and human HB-GAM used to analyse the specificity of our Mabs were kind gifts from Dr. H. Rauvala and from Dr. J. Courty.

Preparation of monoclonal anti-chicken HB-GAM antibodies was performed as previously described for Mabs anti-chicken RIHB (Vigny *et al.*, 1989).

The hybridoma supernatant 13F4 used to specifically localize the embryonic muscles was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA; under contract N01-HD-6-2915 from the NICHD.

SDS-polyacrylamide gel electrophoresis and Western Blot Analysis were performed as previously described (Cockshutt *et al.*, 1993). Briefly, purified chicken HB-GAM (100ng/10 µl of PBS) were incubated in presence of non-reducing electrophoresis sample buffer (10 µl) at room temperature overnight. They were separated on an 18% SDS-polyacrylamide gel. The proteins were transferred to Immobilon-P by a semidry method of electrotransfer (Nova Blot by Pharmacia). The blot was blocked in 5% skimmed milk powder in PBS, incubated with anti-HB-GAM Mabs (5A12 or 6D2) or with an anti-RIHB monoclonal antibody (4C7) or rabbit affinity purified polyclonal antibodies to rat HB-GAM in 0.5% milk in PBS-0.05% Tween 20. It was then incubated with biotinylated anti-mouse IgG antibodies (1/2000) or with biotylinated anti-rabbit IgG antibodies (Amersham), and with streptavidin-conjugated horseradish peroxidase (1/500) (Amersham). ECL detection system (Amersham) was used to expose autoradiographic films.

In situ hybridization

Chicken *HB-GAM* cDNA (160bp) (Nolo *et al.*, 1996) subcloned in pCRII was a kind gift of Dr Rauvala. Patterns of gene transcription were determined using Digoxygenin labelled antisense and sense RNA probes (Boehringer Mannheim) following the protocol described by Strahle *et al.* (1994) and modified by Myat *et al.* (1996) with one modification: the hybridization was done at 60°C.

Immunocytoperoxidase staining

Total embryos or chick embryonic legs were fixed either as described in the *in situ hybridization* protocol or with 4% paraformaldehyde in Phosphate buffer (0.1M, pH 7.4) during 2 h at room temperature. Both procedures led to identical results. Tibias from embryos older than stage 38 (day 12) were decalcified with EDTA 0.2M in PBS pH 7.4 (see Schmid and Linsenmayer, 1985). Further treatments and section of fixed tissues were performed as previously described (Duprez *et al.*, 1993). For embryos older than stage 25-26 (5 days), limb sections were pre-treated with 1mg/ml hyaluronidase in PBS pH 7.2 for 30 min at 37°C to remove cartilage proteoglycan. In the absence of this treatment, as previously found for RIHB (Duprez *et al.*, 1993), the staining was much less intense, suggesting that HB-GAM epitopes were masked or trapped in the cartilaginous matrix. Conversely this treatment did not change the HB-GAM immunostaining in embryos younger than 5 days. Sections were incubated for 30 min with a mixture of 5% skimmed milk powder, 50mM NH4Cl (to block unspecific sites) and 0.5% H_2O_2 (to inactivate endogenous peroxidases) in PBS and incubated for 2 h with specific antibodies diluted in PBS containing 1% skimmed milk powder at room temperature. After washing, they were incubated for 1 h with sheep anti-rabbit IgG or anti-mouse IgG conjugated to peroxidase (Sanofi Pasteur, France) at room temperature. The peroxidase activity was revealed using Sigma Fast DAB and the sections were counterstained with hemalun blue solution.

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