Ovotransferrin and Ovotransferrin Receptor Expression during Chondrogenesis and Endochondral Bone Formation in Developing Chick Embryo

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Abstract. Ovotransferrin expression during chick embryo tibia development has been investigated in vivo by immunocytochemistry and in situ hybridization. Ovotransferrin was first observed in the 7 day cartilaginous rudiment. At later stages, the factor was localized in the articular zone of the bone epiphysis and in the bone diaphysis where it was concentrated in hypertrophic cartilage, in zones of cartilage erosion and in the osteoid at the chondro-bone junction. When the localization of the ovotransferrin receptors was investigated, it was observed that chondrocytes at all stages of differentiation express a low level of the oviduct (tissue) specific receptor. Interestingly, high levels of the receptor were detectable in the 13-d old tibia in the diaphysis collar of stacked-osteoprogenitor cells and in the layer of derived osteoblasts. High levels of oviduct receptor were also observed in the primordia of the menisci.

Metabolic labeling of proteins secreted by cultured chondrocytes and osteoblasts and Northern blot analysis of RNA extracted from the same cells confirmed and completed the above information. Ovotransferrin was expressed by in vitro differentiating chondrocytes in the early phase of the culture and, at least when culture conditions allowed extracellular matrix assembly, also by hypertrophic chondrocytes and derived osteoblast-like cells. Osteoblasts directly obtained from bone chips produced ovotransferrin only at the time of culture mineralization. By Western blot analysis, oviduct receptor proteins were detected at a very low level in extract from differentiating and hypertrophic chondrocytes and at a higher level in extract from hypertrophic chondrocytes undergoing differentiation to osteoblast-like cells and from mineralizing osteoblasts.

Based on these results, the existence of autocrine and paracrine loops involving ovotransferrin and its receptor during chondrogenesis and endochondral bone formation is discussed.

ENDOCHONDRAL bone formation during embryo development is characterized by the formation of hypertrophic cartilage followed by its calcification, erosion, invasion by blood vessels, and substitution by bone tissue. It has been generally believed that chondrocytes of the hypertrophic cartilage are terminally differentiated cells whose ultimate fate is death. Nevertheless hypertrophic chondrocytes in organ cultures start expressing proteins considered to be bone specific markers (Richman and Diewert, 1988; Strauss et al., 1990; Thesingh et al., 1991). We have reported that chick growth plate hypertrophic chondrocytes, when transferred to anchorage dependent cultures in the presence of ascorbic acid, further differentiate to osteoblast-like cells (Descalzi Cancedda et al., 1992; Gentili et al., 1993). This differentiation process is characterized by resumption of proliferative activity, switch from the synthesis of type II and X collagen and other cartilage specific extracellular matrix macromolecules to synthesis of type I collagen and bone-specific proteins, alkaline phosphatase expression and eventually deposition of calcium mineral on the newly formed matrix. This process is enhanced by retinoic acid and is strictly dependent upon cell-substratum interactions. More recently we have proposed that differentiation of hypertrophic chondrocytes toward an osteoblast-like phenotype may occur also in vivo underneath early/prospective periosteum (Galotto, M., G. Campanile, G. Robino, F. Descalzi Cancedda, P. Bianco, and R. Cancedda, manuscript submitted for publication). In the course of these studies, we have shown that hypertrophic chondrocytes differentiating in vitro

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to osteoblast-like cells express relatively large amounts of an 82-kD glycoprotein, subsequently identified as ovotransferrin, a member of the transferrin family (Gentili et al., 1993).

Transferrins are monomeric, glycosylated, 80 kDa proteins with the property of reversibly binding iron (de Jong et al., 1990). Liver is the major site of transferrin production in vertebrates and hepatocyte the main cell involved in its synthesis. Other cells synthesizing transferrin are Sertoli cells (Skinner et al., 1989), oviduct cells (McKnight et al., 1980) and, in the brain, ependymal cells and oligodendroglial cells (Tsutsumi et al., 1989; Bloch et al., 1985). Transferrins are characterized by a large heterogeneity and microheterogeneity due to variations in the polypeptide chain and linked glycan chains and to differences in iron content. It is widely accepted that the major role of transferrin is iron transport and delivery to cells and tissues, nevertheless it has also been proposed that transferrin is important for growth and differentiation of several cell types. In particular, transferrin seems to play a significant role as a mitogenic factor (Trowbridge and Omary, 1981), as a neuro- (Aizenman et al., 1986) and myotrophic agent (Li et al., 1982) and in embryonic morphogenesis (Ekblom et al., 1983; Partanen et al., 1984).

By immunolocalization and in situ hybridization, in the present report we have deeply investigated the expression of ovotransferrin in the developing chick embryo tibia in vivo. The results obtained are corroborated by additional observations made by studying the expression of the factor in cultured chondrocytes and osteoblasts. The expression of ovotransferrin receptors was also investigated. Based on our results, the occurrence of autocrine and paracrine loops of ovotransferrin and its receptor during chondrogenesis and endochondral bone formation may be postulated.

Materials and Methods

Immunohistochemistry

For ovotransferrin and oviduct ovotransferrin receptor immunolocalization, deparaffinized sections of limbs of day 7, 9, 10, and 13 chick embryos were treated with methanol:H_2O_2 (49:1) for 30 min to inhibit endogenous peroxidases, then the sections were treated with 1 mg/ml of hyaluronidase in PBS for 20 min at 37°C, washed in PBS, and exposed to undiluted normal goat serum for 20 min at room temperature. After thorough washing in PBS, the sections were exposed to the specific antiserum for 1 h at room temperature and, after additional washing, incubation was continued with a biotinylated-goat anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc. West Grove, PA; diluted 1:200 in PBS, 0.1% BSA) for 30 min at room temperature.

To detect sites of antibody binding, sections were challenged with peroxidase-conjugated egg-white avidin (Jackson Immunoresearch Laboratories Inc.). Sections were then washed in PBS and 50 mM Na acetate pH 5 and the peroxidase activity was visualized during a 15-min incubation in the dark at room temperature by the enzymatic modification of the 3-amino-9-ethylcarbazole substratum (1 ml of a 0.4% solution of 3-amino-9-ethylcarbazole in dimethylformamide; 9 ml of 50 mM Na acetate pH 5; 0.01 ml of 30% H_2O_2). Sections were then counterstained with Harris' hematoxylin and mounted with Gel/mount from Biomeda Corp (Foster City, CA). Sections were studied and photographed in a Zeiss Axiophot (Oberkochen, Germany).

Antibodies. An antiserum directed against chicken ovotransferrin was obtained in our laboratory by injecting a rabbit with the protein purified from conditioned culture medium as described by Descalzi Cancedda et al. (1992). Before use the antiserum was affinity purified on commercially available ovotransferrin (Sigma Chem. Co.) bound to Sepharose 4B-CNBr (Pharmacia LKB Biotechnology, Uppsala, Sweden). The antiserum against the chick oviduct ovotransferrin receptor was kindly provided by Dr. John J. Lucas (Poola and Lucas, 1988; Fuerkranz et al., 1991). The author stated that the antiserum used for the published experiments was no longer available; continuing to inject the same rabbit with antigen, antisera preparations detected on Western blots few more bands, in addition to the doublet of ~96 kD characteristic of the oviduct transferrin receptor. We therefore preliminarily tested the antibodies specificity by immunoblot experiments with chondrocyte extracts (see Results). Monoclonal antibodies (IgG1, k chain) specific for the chick embryo red blood cell receptor were prepared in the lab of Dr. Anne B. Mason and kindly provided to us before publication.

Preparation of the Probe for In Situ Hybridization

The template used to synthesize the single stranded RNA complementary to ovotransferrin was from ovotransferrin cDNA (Jeltsch and Chambon, 1982). Probes, labeled with ^{35}S-UTP to specific activity of 10^8 cpm/μg, were synthesized using SP6 or T7 polymerase. The 440-bp long probes were used after alkaline hydrolysis to reduce the size to ~150 bp and after selective ethanol and ammonium acetate precipitation to eliminate the unincorporated label.

In Situ Hybridization

Slides were dewaxed and passed sequentially through 10 mg/ml proteinase K, 4% paraformaldehyde for 5 min, 1/400 (vol/vol) acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 min followed by addition of the hybridization mixture. Hybridization buffer was 50% deionized formamide, 10% dextran sulfate, 0.3 M NaCl, 20 mM Tris-HCl, pH 7.6, 5 mM EDTA, Denhart solution 1×, 0.5 mg/ml of tRNA, and 10 mM DTT containing 1.5×10^5 cpm/μl of the appropriate probe. After hybridization overnight at 50°C, slides were rinsed for 2 h at 50°C in hybridization buffer, digested with 20 μg/ml RNAse A for 30 min at 37°C and then washed again, dehydrated, and dried in air. Slides were then dipped in NTB-2 emulsion (diluted 1:1 in water) and exposed for 12 d at 4°C, after which time they were developed, fixed, and mounted with DPX mountant.

Cell Culture

Cultures of chondrocytes were performed as described (Castagnola et al., 1986). Cells derived from 6-d chick embryo tibiae, expanded as adherent dedifferentiated cells for 2 wk, were transferred to suspension culture for 3–4 wk until a homogeneous population of single isolated hypertrophic chondrocytes was visible. To obtain osteoblast-like cells as described (Descalzi Cancedda et al., 1992), hypertrophic chondrocytes were filtered through a nylon filter Nitex 42 μm mesh in order to remove cells still aggregated, digested with hyaluronidase (1 mg/ml), and plated (2×10^5 in 35-mm dish) in Coon's modified F12 culture medium (Ambesi Impiombato et al., 1980) supplemented with 10% FCS. After 3 d the medium was supplemented with 100 μg/ml ascorbic acid and 10 mM β-glycerophosphate (complete medium). Culture was continued without cell passaging. When indicated retinoic acid was added to the culture medium at 500 nM final concentration. Fresh retinoic acid was added and the culture medium was changed every day.

Cultures of chick embryo osteoblasts were obtained from cells outgrown from bone chips according to Manduca et al. (1992).

Cell Labeling and Protein Analysis

Cells were labeled with [^{35}S]methionine as described (Descalzi Cancedda et al., 1988). Samples of culture media were run for protein analysis on SDS-PAGE in reducing and unreducing conditions (Bonatti and Descalzi Cancedda, 1982). Immunoprecipitation of specific proteins was performed as in Descalzi Cancedda et al. (1988).

Western Blot Analysis

Cell lysates were prepared by adding 0.1% SDS in PBS to the cells. Samples, containing ~300 μg of proteins, were applied to a 10% SDS-polyacrylamide gel. Electrophoresis was performed in reducing conditions. After electrophoresis, the gel was blotted to a BA85 nitrocellulose membrane (Schleicher and Schuell GmbH, Dassel, Germany) as described by Towbin et al. (1979). After saturation for 16 h with 2% BSA in TTBS buffer (20 mM Tris HCl pH 7.5, 500 mM NaCl, 0.05% Tween 20), the blot was incubated for 2–16 h at room temperature either with the polyclonal antiserum directed against the oviduct transferrin receptor or with the monoclonal antibodies directed against red blood cell transferrin receptor. After washing, bound antibodies were detected by a biotin conjugated anti-rabbit IgG

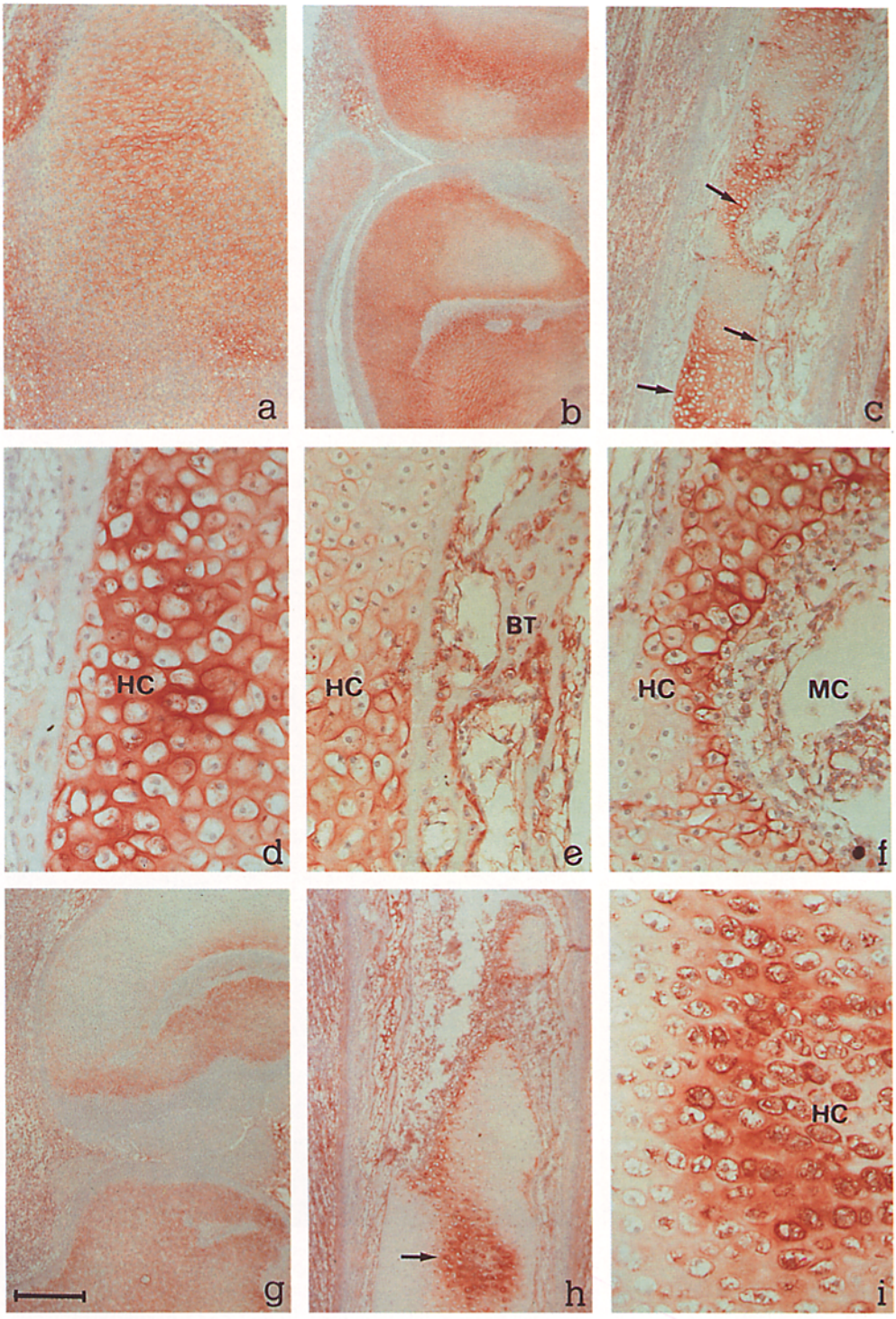

Figure 1. Immunolocalization of ovotransferrin in the developing chick embryo tibia. Sections of 7 day (*a*), 10 day (*b–f*), and 13 day (*g–i*) tibiae were stained with ovotransferrin antiserum. *b* and *g* refer to the epiphysis region. *c–f* and *h–i* refer to the diaphysis region. *d–f* and *i* are enlargement of regions indicated by arrows in *b* and *h*. Ovotransferrin is present in the cartilaginous rudiment at day 7 and epiphysis of early embryos; at later stages, it is associated with frank chondrocytes in the hypertrophic cartilage and by cells (possibly stage III chondrocytes and osteocytes) within the first bone at the chondro-bone junction. *HC*, hypertrophic chondrocyte; *MC*, marrow cavity; *BT*, bone trabecule. Bars: (*A*) 100 μm; (*B–C, G–H*) 200 μm; (*D–F, I*) 50 μm.

(H + L) (Jackson Immunoresearch Laboratories Inc.) and avidine coupled-horseradish peroxidase (Jackson Immunoresearch Laboratories Inc.) using 4-chlor-1-napthol (Merck Darmstadt, Germany) as substrate. Antibody and avidine-peroxidase solutions were made in TTBS containing 2% BSA.

RNA Extraction and Northern Analysis

Total RNA was extracted from cells using the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). For Northern analysis ~10 μg of total RNA, electrophoresed through 1% agarose gel in the presence of formaldehyde, was blotted onto Hybond-N-membranes. Hybridization and washing conditions were as recommended by Amersham Corp. Probes were BST XI-BST XI fragment of ovotransferrin cDNA (Jeltsch and Chambon, 1982); Eco RI-Eco RI fragment of clone CHKCTR3 for red blood cell receptor cDNA (Chan et al., 1989; Gerhardt et al., 1991); and pXCR7 for rRNA (gift from Dr. F. Amaldi, Universitá di Tor Vergata, Roma, Italy).

Results

Immunolocalization of Ovotransferrin in the Developing Tibia

To investigate ovotransferrin expression in vivo, sections of tibiae from 7–13-d embryos were stained with antibodies

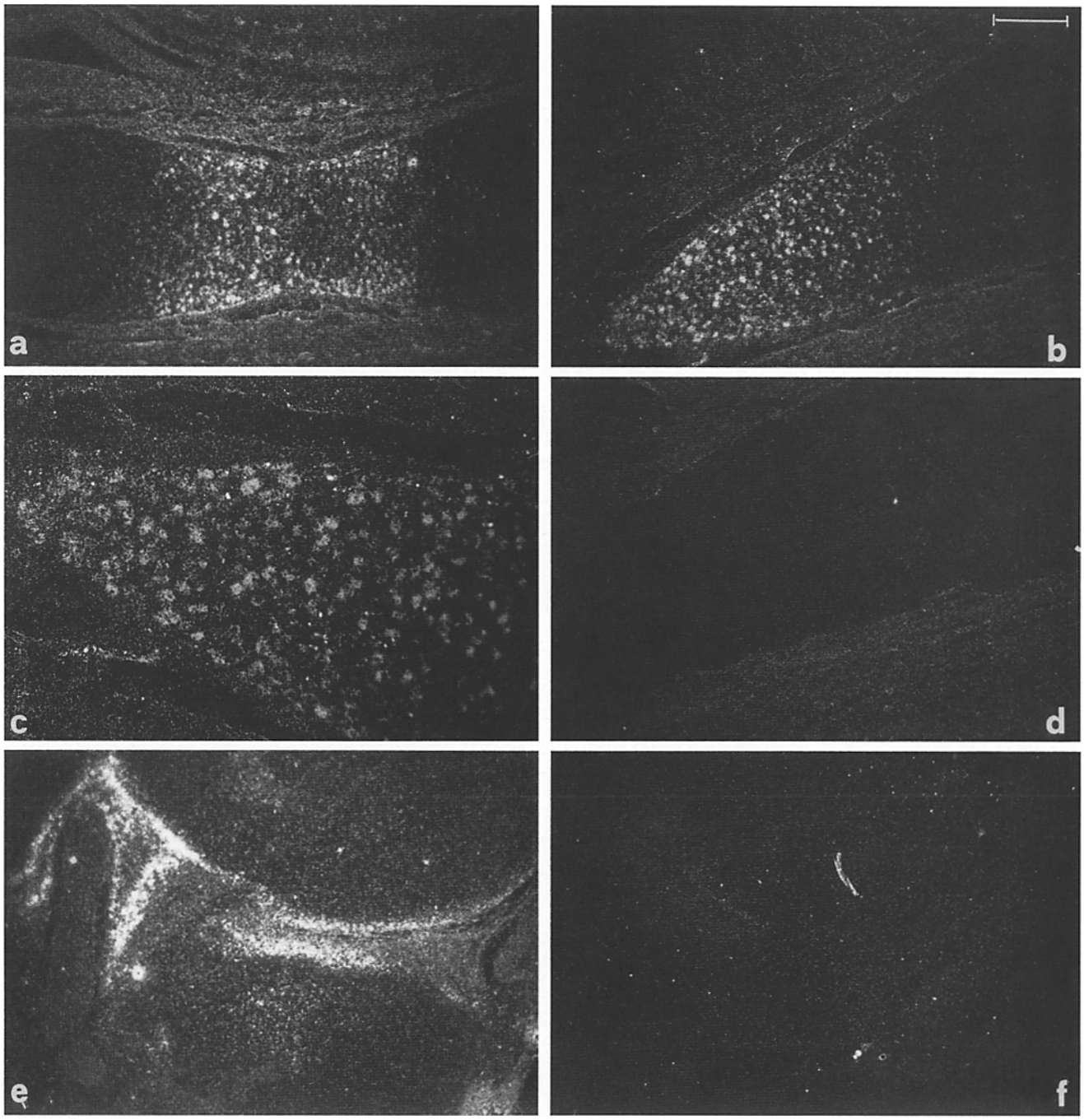

Figure 2. Localization of ovotransferrin mRNA in the developing chick embryo tibia. In situ hybridization was performed on sections of 13 day (*A–D*) and 10 day (*E–F*) hindlimb with ovotransferrin antisense (*A–C, E*) and sense (*D* and *F*) riboprobes. In *A* is shown a detail of a digit cartilage rudiment. Other panels refer to diaphysis (*B–D*) and epiphysis (*E–F*) of the tibia. Bars: (*A–B, D–F*) 200 μm; (*C*) 100 μm.

raised against the purified protein. Ovotransferrin was detected in the cartilaginous rudiment of day 7 embryos (Fig. 1 *a*). During development the ovotransferrin was readily identified at early stages of development in the tibia epiphyseal regions (Fig. 1 *b*), but progressively diminished and remained restricted to areas immediately adjacent to articular surface (Fig. 1 *g*). In the bone diaphysis ovotransferrin was located in hypertrophic cartilage and in osteoid at the chondro-osseous junction of both 10 (Fig. 1, *c–f*) and 13- (Fig. 1, *h–i*) d old embryos. Especially at later stages of development, the ovotransferrin was not uniformly distributed in the hypertrophic cartilage and was more concentrated in the central part and in areas of cartilage erosion. In the osteoid at the chondro-bone junction, elongated cells trapped within the extracellular matrix presented a frank positivity (Fig. 1 *e*); these positive cells were located in both the carti-

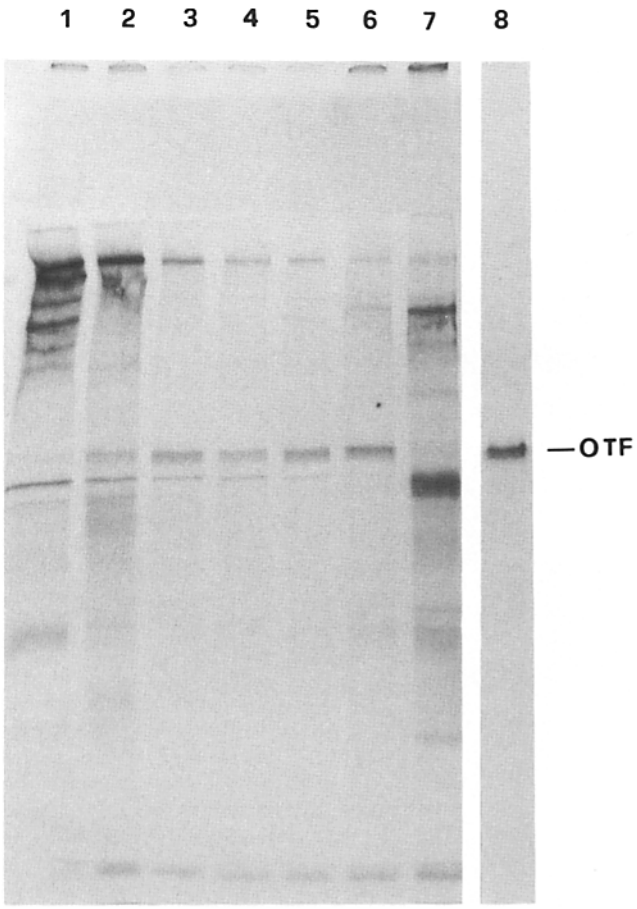

Figure 3. Protein secreted by in vitro differentiating chondrocytes. Aliquots of [^{35}S]methionine-labeled culture media from dedifferentiated chondrocytes grown as adherent cells (lane *1*) and transferred to suspension culture for 1 day (lane *2*), 2 days (lane *3*), 3 days (lane *4*), 4 days (lane *5*), 7 days (lane *6*), and 2 wk (lane *7*) were analyzed on 12.5% polyacrylamide gel. In lane *8* an aliquot of labeled culture medium equivalent to the one analyzed in lane *6* was immunoprecipitated with specific anti-ovotransferrin antiserum. Culture medium was not supplemented with ascorbic acid. Electrophoresis was performed in reducing conditions. OTF refers to ovotransferrin.

lage and the bone side of the first osteoid deposited and were tentatively identified as osteoblast-like cells (Stage III chondrocytes) and osteocytes.

Localization of Ovotransferrin mRNA by In Situ Hybridization

In situ hybridization on paraffin embedded sections of 10- and 13-d old hindlimbs was performed to analyze the ovotransferrin mRNA distribution in the forming bones. Similar results were obtained when the sections from the 10- and the 13-d old embryos were analyzed. In Fig. 2 *A* is shown a detail of a digit cartilage rudiment from the 13-d old embryo. Due to the different timing of limb bone development, this image represents an earlier development stage than the one of tibia in the same embryo. Other panels refer to diaphysis (*B–D*) and epiphysis (*E–F*) of the 13- and 10-d old tibia. A strong hybridization signal was displayed by the hypertrophic chondrocytes, but not by the more immature chondrocytes (*A–C*). In the epiphyseal region, also the prearticular cartilage presented a strong positive signal (*E*). As expected no signal was detected when the hybridization was performed with the control sense riboprobe (*D* and *F*).

Expression of Ovotransferrin by In Vitro Differentiating Chondrocytes: Importance of Cell-Extracellular Matrix Interactions

We have previously shown production of ovotransferrin by cultured chondrocytes undergoing differentiation to osteoblast-like cells (Descalzi Cancedda et al., 1992; Gentili et al., 1993). We therefore focused our attention on the initial phase of the in vitro chondrogenesis. Labeled culture media were collected from cultures of dedifferentiated chondrocytes grown as adherent cells and transferred and maintained for different time in suspension culture. Aliquots of media were analyzed on SDS polyacrylamide gel (Fig. 3). Ovotransferrin was identified based on the different electrophoretic mobility in reducing and non-reducing conditions (not shown) and on its immunoprecipitability by specific antibodies. Ovotransferrin was not produced by dedifferentiated cells, was secreted by differentiating chondrocytes during the first week of the in vitro chondrogenesis, but it was absent in conditioned media from hypertrophic chondrocytes after 2 wk suspension culture.

This last observation was in apparent contrast with the presence of ovotransferrin in the hypertrophic cartilage zone of embryo tibiae (Fig. 1). Therefore we reasoned that the lack of ovotransferrin production by cultured hypertrophic chondrocytes might be due to the absence of a properly organized extracellular matrix (obviously present in vivo) in our standard culture conditions due to the absence of ascorbic acid in the medium. In fact, only in the presence of ascorbic acid the collagen chains are correctly hydroxylated and the extracellular matrix fully assembled. To test this hypothesis we performed one experiment in which a population of dedifferentiated chondrocytes, split at the time it was transferred to suspension culture, was maintained either in the presence or it the absence of ascorbic acid. Culture media from 9- and 13-d culture were analyzed (Fig. 4 *A*). Only when the culture medium was supplemented with ascorbic acid, ovotransferrin was secreted by the cells.

In another series of experiments, hypertrophic chondrocytes were obtained after 3 wk suspension culture in the absence of ascorbic acid. At that time the chondrocyte population was divided and the suspension culture was continued for 5 and 20 additional days either in the presence or in the absence of ascorbic acid (Fig. 4 *B*). Also in this case the presence of ascorbic acid in the culture medium was an absolute requirement for the production of ovotransferrin.

Northern blot analysis of mRNAs extracted from chondrocytes confirmed the activation of the ovotransferrin gene transcription in the initial phase of the chondrogenesis (Fig. 5 *A*). After 12 d the signal, although at low level, was present only in chondrocytes cultured in the presence of ascorbic acid (Fig. 5 *B*), in agreement with the observed presence of the protein after 13 d (Fig. 4 *A*). Ovotransferrin mRNA was induced by retinoic acid in the presence of ascorbic acid in hypertrophic chondrocytes replated as adherent cells (Fig. 5 *C*).

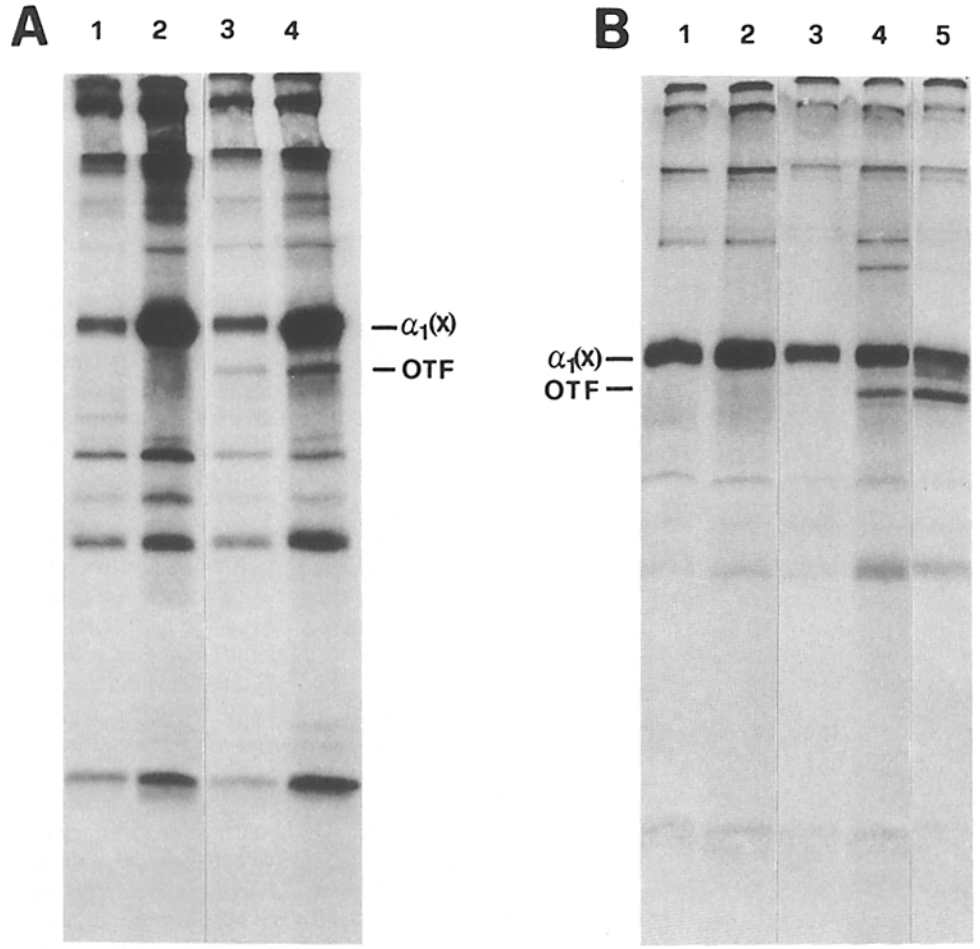

Figure 4. Extracellular matrix assembly and ovotransferrin production by cultured chondrocytes. Aliquots of [^{35}S]-methionine-labeled culture media were analyzed on 12.5% polyacrylamide gel. (*A*) Dedifferentiated chondrocytes transferred to suspension culture and maintained in culture medium non supplemented (lanes *1–2*) or supplemented (lanes *3–4*) with ascorbic acid for 9 (lanes *1* and *3*) or 13 d (lanes *2* and *4*). (*B*) Hypertrophic chondrocytes grown in suspension culture for 3 wk in medium non supplemented with ascorbic acid (lane *1*), filtered, and maintained for 5 additional days (lanes *2* and *4*) and 20 d (lanes *3* and *5*) in medium non-supplemented with ascorbic acid (lanes *2–3*) and supplemented (lanes *4–5*) with ascorbic acid. Electrophoresis was performed in non-reducing conditions. OTF refers to ovotransferrin.

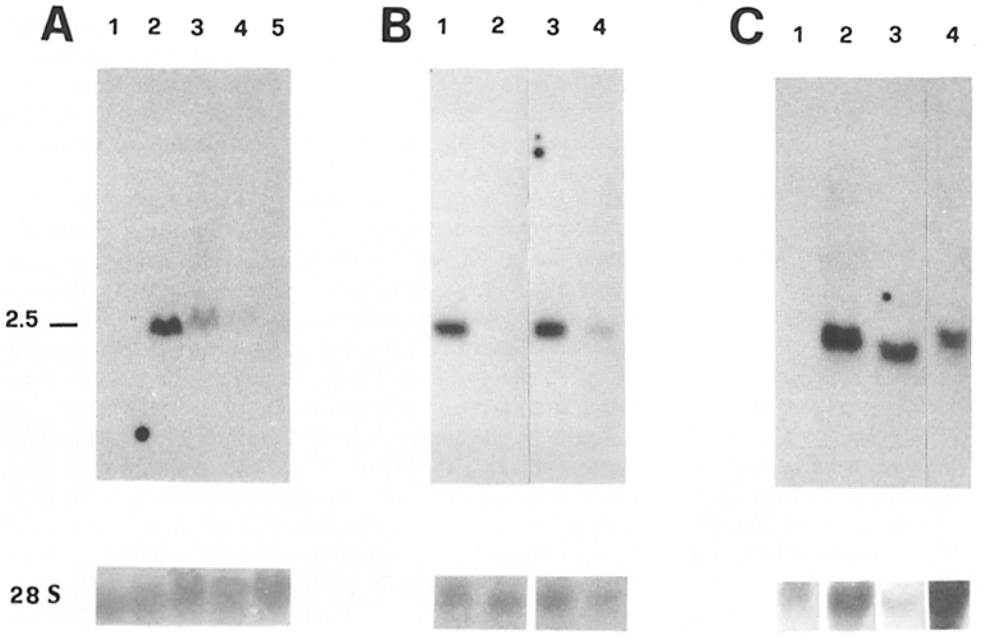

Figure 5. Northern blot of RNAs extracted from cultured chondrocytes. (*A*) Dedifferentiated chondrocytes grown as adherent cells (lane *1*) were transferred to suspension culture for 36 h (lane *2*), 3 d (lane *3*), 11 d (lane *4*), and 3 wk (lane *5*) without ascorbic acid. (*B*) Dedifferentiated chondrocytes were transferred to suspension culture for 3 days (lanes *1* and *3*) and 12 days (lanes *2* and *4*); culture medium was either not supplemented (lanes *1–2*) or supplemented (lanes *3–4*) with ascorbic acid. (*C*) Hypertrophic chondrocytes grown in suspension for 4 wk (lane *1*), replated as adherent cells and grown in the presence of ascorbic acid and retinoic acid for additional 3 (lane *2*), 7 (lane *3*), and 15 d (lane *4*). At the bottom the 28 S rRNA region of the same filter after rehybridization with the probe for rRNA is shown. Probes were as follows: BST XI-BST XI fragment of ovotransferrin cDNA, pXCR7 for rRNA. Number on the left refers to RNA size in kb.

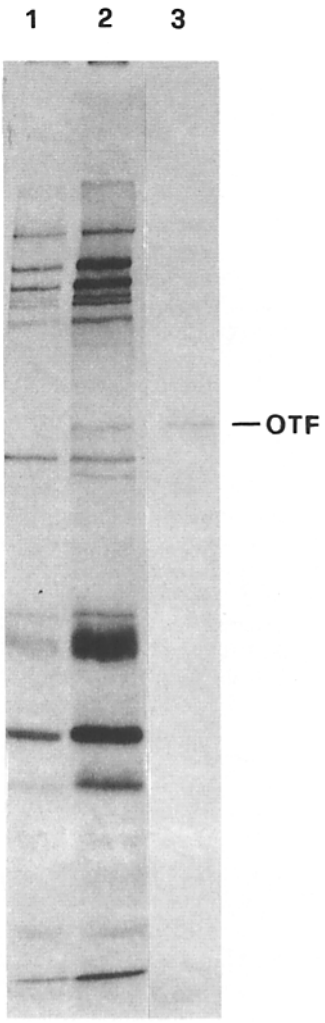

Figure 6. Production of ovotransferrin by mineralizing osteoblasts. Osteoblasts grown out from bone chips were cultured for 15 (lane *1*) and 39 d (lane *2*) in the presence of ascorbic acid. Frank areas of mineralization were detectable in the culture after 39 d, but not after 15 d. Aliquots of [^{35}S]methionine-labeled culture media were analyzed on 12.5% polyacrylamide gel. In lane *3*, an aliquot of labeled culture medium equivalent to the one analyzed in lane *2* was immunoprecipitated with specific anti-ovotransferrin antiserum. Electrophoresis was performed in non-reducing conditions. OTF refers to ovotransferrin.

Expression of Ovotransferrin by In Vitro Mineralizing Osteoblasts

Osteoblasts grown out from bone chips were maintained in culture at confluence for 15 d and 39 d. At variance with the 15-d culture, after 39 d the culture was heavily mineralized, as revealed by microscopic observations and von Kossa staining (not shown). Immunoprecipitable ovotransferrin was produced by the mineralizing osteoblasts, but not by the osteoblasts at an earlier stage of culture (Fig. 6). In other cultures that underwent mineralization after a different number of days, ovotransferrin production was observed only at the time of the culture mineralization.

Expression of Ovotransferrin Receptors in Cultured Chondrocytes and Osteoblasts

Two different receptors for ovotransferrin have been described in chicken cells: one specific for chick embryo erythroid cells (Schmidt et al., 1985, 1986) and one identified in the hen oviduct (Poola and Lucas, 1988; Fuerkranz et al., 1991). Recombinant clones of the chicken red blood cell ovotransferrin receptor gene and cDNA have been isolated and sequenced (Chan et al., 1989; Gerhardt et al., 1991) and the developmental pattern of expression of the red blood cell receptor has been investigated by Northern blot in various chick embryonic tissues (Chan and Gerhardt, 1992). The receptor mRNA is present at significantly higher levels (from 20 times to 200 or more times) in erythroid cells than in nonerythroid cells and decline during embryonic development in all tissues, reaching undetectable levels in all of the nonerythroid tissues by hatching. Monoclonal antibodies specific for the chick embryo red blood cell receptor are available. For the oviduct transferrin receptor, a polyclonal antiserum directed against this receptor has been described (Poola and Lucas, 1988). The antiserum strongly stained adult oviduct, ovary and liver, but not red blood cells either from embryos or from adult chicken (Fuerkranz et al., 1991).

When the RNAs extracted from cultured chondrocytes were analyzed by Northern blot using the erythroid ovotransferrin receptor cDNA (not shown), the probe showed high level of ovotransferrin mRNA in the RNA extracted from embryonic liver and identified a barely detectable mRNA of the correct size in the RNAs from chondrocytes but not from dedifferentiated and osteoblast-like cells. Similar results were obtained by RNase protection experiments (Castagnola, P., unpublished observations).

To investigate the presence of receptor proteins and, at the same time, to test antibody specificities, we preliminarily performed immunoblot experiments on cell lysates from chondrocytes and osteoblasts cultured in different conditions. At the protein concentration loaded on the gel (300 μg), the monoclonal antibodies directed against the red blood cell receptor recognized the characteristic 95-kD protein in an embryonic liver extract, but failed to identify any protein in all samples from cultured cells (not shown). Positive results were obtained with the antiserum recognizing the oviduct receptor (Fig. 7). The antibodies recognized in adherent dedifferentiated cells and in chondrocytes cultured in suspension a doublet of 95 kD comigrating with the bands detected in hen oviduct microsomes (Fig. 7 *A*). The number of receptor molecules in the cell lysates highly increased during the differentiation of hypertrophic chondrocytes to osteoblast-like cells promoted by retinoic acid (Fig. 7 *B*). Comparable amount of receptor was present in lysates from mineralizing osteoblasts (Fig. 7 *C*). The anti-oviduct receptor serum, in addition to the characteristic 95-kD doublet, recognized few more minor bands of higher molecular weight.

Expression of Ovotransferrin Receptors in the Developing Tibia

Sections of tibiae from 7-, 9-, and 13-d embryos were stained with the antiserum raised against the oviduct ovotransferrin receptor and monoclonal antibodies against the red blood cell receptor. With the antiserum against the oviduct receptor, a low positivity was observed in the cartilaginous rudiment at day 7 (Fig. 8 *a*) and in both the epiphysis and the diaphysis of the 9-d bone (Fig. 8, *b–c*). In the 13-d bone, epiphyseal cells express only low levels of receptor (Fig. 8 *d*); a very strong staining was instead observed in the diaphysis at the chondro-bone junction (Fig. 8, *e–f*). At a higher magnification it is evident that the highly positive cells are located in the collar of stacked-osteoprogenitor-cells and in the layer of derived osteoblasts (Fig. 8 *f*). It is interesting to note that at 13 day also the primordia of the menisci

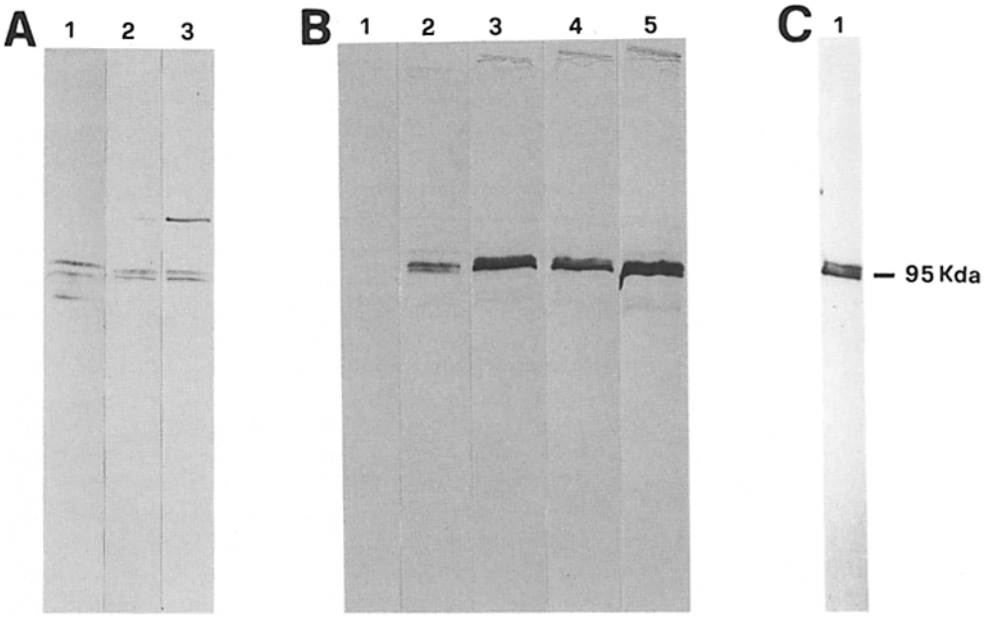

Figure 7. Immunoblot analysis of transferrin receptor. Aliquots of cell lysates containing 300 μg proteins were run on SDS-polyacrylamide gel, blotted to nitrocellulose filter and subjected to immunoblot with antiserum directed against oviduct ovotransferrin receptor. (*A*) Dedifferentiated chondrocytes grown as adherent cells (lane *2*) and transferred to suspension culture for 15 d (lane *3*). In lane *1* an aliquot of hen oviduct microsomes (a gift of Dr. J. J. Lucas) is run for comparison. (*B*) Hypertrophic chondrocytes replated as adherent cells and treated with 500 nM retinoic acid for 3 (lane *1*), 6 (lane *2*), 10 (lane *3*), 13 (lane *4*), and 27 (lane *5*) d. Mineral deposits were observed in the cell layer at the last time of the culture. (*C*) Osteoblasts maintained in culture for 39 d, a time when mineralization has already occurred.

is stained by the anti-ovotransferrin receptor antibodies (Fig. 8 *d*).

In the 13-d embryo, when the antibodies directed against the red blood cell receptor were used, only a faint peroxidase activity was observed in the epiphysis and in the hypertrophic cartilage zone. Enrichment of this receptor was never observed in osteoprogenitor cells and derived osteoblasts, as in the case of the oviduct receptor (not shown).

Discussion

In this study we have investigated the ovotransferrin expression during development of the chick embryo tibia. Ovotransferrin is present in the first cartilaginous rudiment. While development continues, in the epiphyseal region, ovotransferrin decreases and remains restricted to the articular zone. In the diaphysis the factor, which is abundant in the hypertrophic cartilage, progressively concentrates in zones of cartilage erosion and in the osteoid at the chondro-bone junction. The studies with cultured chondrocytes and osteoblasts corroborated the morphological observations. In culture, ovotransferrin was expressed by the in vitro differentiating chondrocytes in the initial phase of the culture and by hypertrophic chondrocytes in the presence of ascorbic acid. Ovotransferrin is also expressed by hypertrophic chondrocytes undergoing differentiation to osteoblast-like cells and by mineralizing osteoblasts. The two last processes occur in the presence of ascorbic acid.

Two types of transferrin receptors have been described in chick cells. One is expressed in cells of the erythroid lineage (Schmidt et al., 1985, 1986), the other is known as the oviduct receptor, since was first identified in oviduct cells (Poola and Lucas, 1988), but could be better defined as the tissue type transferrin receptor since it is expressed in several tissues (Fuerkranz et al., 1991; our work in this manuscript). When the presence of the ovotransferrin receptors was investigated in the developing tibia, it was found that, at all developmental stages, the oviduct receptor, was expressed at a low level and almost evenly distributed in all cartilages. High level of oviduct receptor expression was observed only in the 13 day tibia, in the collar of stacked-osteoprogenitor-cells, and in the layer of derived osteoblasts. The Western blot analysis on the extracts from cultured chondrocytes and osteoblasts were in agreement with the immunolocalization experiments. Low levels of oviduct receptor were detectable in almost all samples but high levels of receptor expression were reached only in extracts from hypertrophic chondrocytes differentiating to osteoblast-like cells and from mineralizing osteoblasts.

Since hypertrophic and differentiating chondrocytes coexpress the ovotransferrin factor and its receptor, the existence of autocrine loops playing a role in the control of chondrogenesis and hypertrophy could be postulated. At late stage of bone development high expression of the receptor is observed in the osteogenic collar surrounding the hypertrophic cartilage. Since the cells in the osteogenic collar do not express the ovotransferrin, a paracrine loop leading to the first bone deposition, as part of the interplay between chondrocytes and osteogenic cells, should be considered. In principle chondrocyte specific factors, as ovotransferrin, could participate in the activation of preosteoblastic cell differentiation program. With continuing development, differentiated mineralized osteoblasts start producing ovotransferrin in turn. Therefore, at this stage, an additional autocrine loop leading to the activation of more preosteoblastic cells could be envisaged. In the embryo limbs, at the time the initial car-

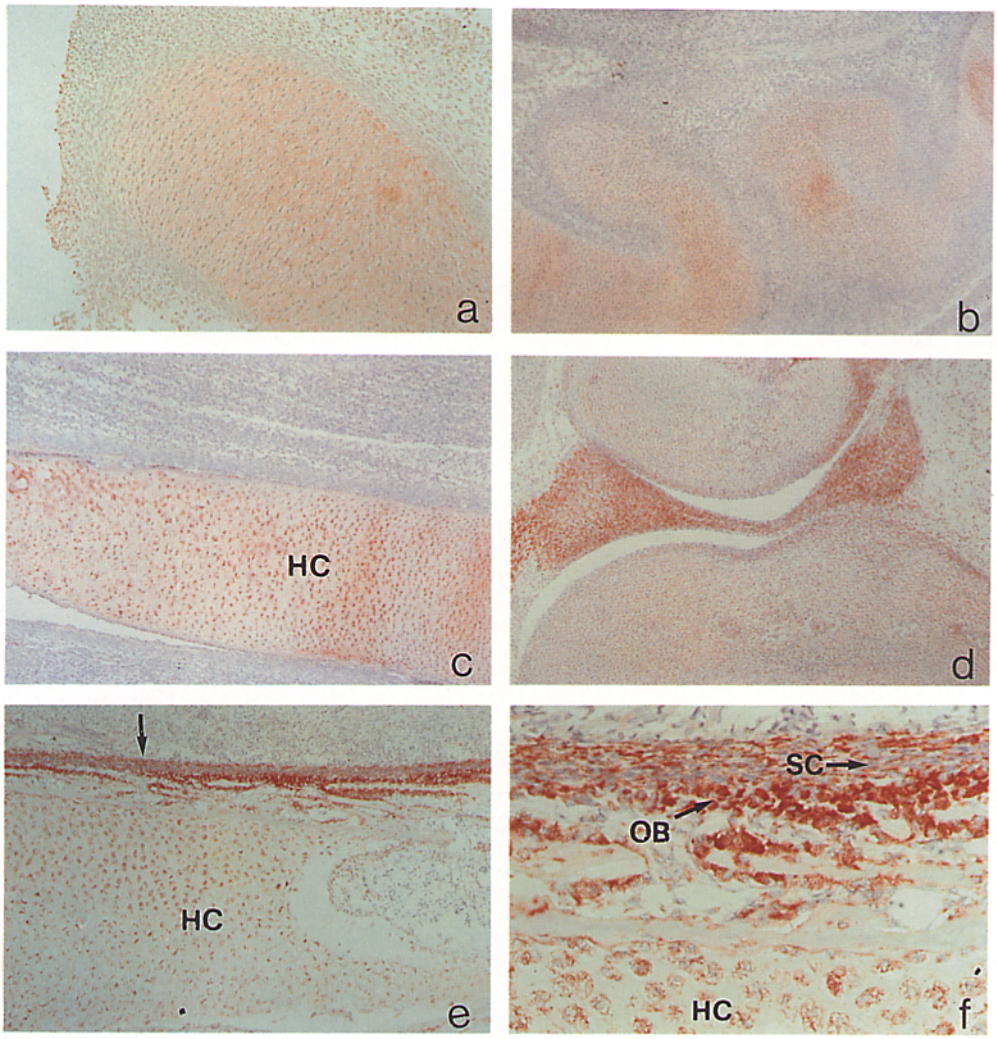

Figure 8. Immunolocalization of ovotransferrin receptor in the developing chick embryo tibia. Sections of 7-d (*a*), 9-d (*b–c*), and 13-d (*d–f*) tibiae were stained with an antiserum directed against the oviduct ovotransferrin receptor. (*b* and *d*) Epiphyseal region of tibia. (*c*, *e–f*) Mid-diaphysis region. Oviduct ovotransferrin receptors are present in the cartilaginous rudiment at 7 days and in all cartilages at other developmental stages, but they are highly enriched only in the 13-d tibia at the osteoblast side of the chondro-bone junction. At 13 day also the primordia of the menisci, which is negative for ovotransferrin (see Fig. 1), is stained by the anti-oviduct-ovotransferrin receptor antibodies. *HC*, hypertrophic chondrocyte; *OB*, osteoblast; *SC*, stacked cell layer. Bars: (*A*) 100 μm; (*B–E*) 200 μm; (*F*) 50 μm.

tilage and bone are formed, blood vessels are not present and the bone rudiment is not vascularized, a local production of ovotransferrin appears to be the only way to make this factor available to the differentiating cartilage and bone cells.

Interestingly high levels of oviduct receptor were observed at 13 day also in the primordia of the menisci. At this developmental stage, these cells do not express ovotransferrin, but, on the contrary, ovotransferrin is detectable in the region of the prearticular cartilage. This finding suggests the existence of paracrine mechanisms also during bone joint morphogenesis.

Ovotransferrin function during the cartilage and bone formation can be only hypothesized. Transferrins reversibly bind iron. Transferrin receptors are expressed at particularly high levels in the erythroid cell lineage between the early and intermediate normoblast stages when the iron requirement is at the highest level; subsequently their number decreases and finally disappears in the mature red blood cells (Newman et al., 1982; Horton, 1983; Chan and Gerhardt, 1992). In addition or in association with their role as iron transporters, transferrins play a role as mitogenic factors. Transferrin receptors are in fact associated with actively proliferating cells, both in vertebrates and invertebrates (Huebers and Finch, 1987). A neuro- and myotrophic activity of transferrin has also been proposed. Transferrin is essential for myotube development in tissue culture (Li et al., 1982; Beach et al., 1985) and is required for the maintenance of the differentiated state of the striated muscle by the nerve (Stamatos and Fine, 1986). A similar situation may occur in brain where the transferrin produced by oligodendrocytes and other cells probably plays a trophic role on developing neurons and astrocytes (Aizenman et al., 1986). Finally a role for transferrin and its receptor in embryonic morphogenesis has been shown by Ekblom et al. (1983) in mouse organ cultures of developing kidneys and teeth (Partanen et al., 1984). Transferrin is necessary to cell proliferation and cell differentiation. According to our results ovotransferrin is expressed in regions of the embryonic cartilage and bone characterized both by cell proliferation and cell differentiation. It should be recalled that often cell differentiation is preceded by a burst of cell proliferation. Therefore, in developing bone ovotransferrin might play a role in the control of both differentiation and proliferation of chondrocytes and osteoblasts.

Ovotransferrin is particularly expressed in regions where tissue morphogenesis occurs. Retinoic acid is a well known morphogenetic factor. Retinoic acid responsive elements have been identified on transferrin promoters (Ham and

Griswald, 1986). We have shown an inductive effect of retinoic acid on ovotransferrin expression in cultured chondrocytes (Descalzi Cancedda et al., 1992; Gentili et al., 1993). Levels of transferrin and transferrin mRNA are responsive to added retinoids also in cultured Sertoli cells (Hugly and Griswold, 1987). It is interesting to note that the Ch21, a protein belonging to the lipocalin family (extracellular transport proteins for small hydrophobic ligands, such as steroids, retinoids, and other small hydrophobic regulatory molecules) (Descalzi Cancedda et al., 1990), is produced by chondrocytes and is localized in the same regions where ovotransferrin is observed (Manduca et al., 1989). We would like to suggest that ovotransferrin is a member of a whole group of proteins coordinately expressed in areas of chondrogenesis and osteogenesis.

The finding that at late stage of development the presence of ascorbic acid, i.e., the assembly of an extracellular matrix, is an absolute requirement for the expression of ovotransferrin by chondrocytes is per se an interesting observation worth additional comments. Ovotransferrin is not the only example where the interaction of the chondrocyte with an organized extracellular matrix is necessary for gene activation. A control over gene expression depending upon the chondrocyte-extracellular matrix interaction has been shown also in the case of the RIHB (Retinoic Induced Heparin Binding factor (Castagnola, P., S. Tavella, M. Gennari, R. van der Werken, P. Raffo, D. Ravlais, M. Vigny, and R. Cancedda, manuscript submitted for publication) and in the case of a newly described angiogenic activity (Descalzi Cancedda, F., A. Melchiori, R. Benelli, C. Gentili, L. Masiello, G. Campanile, R. Cancedda, and A. Albini, manuscript submitted for publication). It therefore appears that, in order to fully express their differentiated phenotype, chondrocytes need to interact with an organized extracellular matrix that they deposit by themselves.

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