Progression and Recapitulation of the Chondrocyte Differentiation Program: Cartilage Matrix Protein Is a Marker for Cartilage Maturation

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During endochondral bone formation, chondrocytes in the cartilaginous anlage of long bones progress through a spatially and temporally regulated differentiation program before being replaced by bone. To understand this process, we have characterized the differentiation program and analyzed the relationship between chondrocytes and their extracellular environment in the regulation of the program. Our results indicate that, within an epiphyseal growth plate, the zone of proliferating chondrocytes is not contiguous with the zone of hypertrophic chondrocytes identified by the transcription of the type X collagen gene. We find that the postproliferative chondrocytes which make up the zone between the zones of proliferation and hypertrophy specifically transcribe the gene for cartilage matrix protein (CMP). This zone has been termed the zone of maturation. The identification of this unique population of chondrocytes demonstrates that the chondrocyte differentiation program consists of at least three stages. CMP translation products are present in the matrix surrounding the nonproliferative chondrocytes of both the zones of maturation and hypertrophy. Thus, CMP is a marker for postmitotic chondrocytes. As a result of the changes in gene expression during the differentiation program, chondrocytes in each zone reside in an extracellular matrix with a unique macromolecular composition. Chondrocytes in primary cell culture can proceed through the same differentiation program as they do in the cartilaginous rudiments. In culture, a wave of differentiation begins in the center of a colony and spreads to its periphery. The cessation of proliferation coincides with the appearance of CMP and eventually the cells undergo hypertrophy and synthesize type X collagen. These results reveal distinct switches at the proliferative-maturation transition and at the maturation-hypertrophy transition during chondrocyte differentiation and indicate that chondrocytes synthesize new matrix molecules and thus modify their preexisting microenvironment as differentiation progresses. However, when “terminally” differentiated hypertrophic chondrocytes are released from their surrounding environment and incubated in pellet culture, they stop type X collagen synthesis, resume proliferation, and reinitiate aggrecan synthesis. Eventually they cease proliferation and reinitiate CMP synthesis and finally type X collagen. Thus they are capable of recapitulating all three stages of the differentiation program in vitro. The data suggest a high degree of plasticity in the chondrocyte differentiation program and demonstrate that the progression and maintenance of this program is regulated, at least in part, by the extracellular environment which surrounds a differentiating chondrocyte during endochondral bone formation.

INTRODUCTION

Cartilaginous rudiments of a developing embryo serve as anlagen from which long bones are formed during endochondral bone formation. During this process the length and shape of a bone is determined. Limb mesodermal precursor cells differentiate into chondrocytes of the cartilaginous growth plate and they progress through a chondrocyte differentiation program, starting as proliferating chondrocytes and ending up as hypertrophic chondrocytes. The hypertrophic chondrocytes are then removed and replaced by bone. The chondrocyte differentiation program has been partitioned into different stages based on cell shape and volume, biosynthetic and metabolic activities, and, most accurately, on molecular markers. At least two models have been proposed. According to one model (Castagnola et al.,...
The differentiation program is divided into two stages and the chondrocytes are considered to be either in the proliferative or in the hypertrophic state. Thus, this model proposes that the proliferative stage immediately precedes the hypertrophic stage during endochondral bone formation. The proliferating chondrocytes synthesize type II collagen and aggregan (Miller, 1972; Doege et al., 1990). These markers distinguish the chondrocytes from their mesodermal precursors which synthesize type I collagen. The hypertrophic chondrocytes produce type X collagen, a hallmark of cartilage hypertrophy, is synthesized (von der Mark et al., 1992). The second model proposes that the chondrocytes of the growth plate are divided into three distinct stages that are identified histologically as the zones of proliferation (zone 1), maturation (zone 2), and hypertrophy (zone 3) (Kim and Conrad, 1977; Stocum et al., 1979). According to this model, the transition of the chondrocytes from the zone of proliferation to the zone of hypertrophy would be separated by an additional developmental stage, the stage of maturation. However, no specific molecular markers have been identified for chondrocytes in this stage.

The molecular markers that characterize the differentiated state of chondrocytes are extracellular matrix (ECM) macromolecules and the chondrocyte differentiation process is modulated by extracellular cues including the ECM (Benya and Schaffer, 1982; von der Mark et al., 1992b). In monolayer culture, chondrocytes which are seeded at low density will transform their chondrogenic phenotype (round shape, type II collagen synthesis) to a fibroblastic one (flattened morphology, type I collagen synthesis) under certain conditions (von der Mark et al., 1977). This transformation depends on cell shape and attachment, which is mediated through cell-matrix interaction (Benya and Schaffer, 1982). The alteration of the chondrocyte differentiation process also occurs in vivo. For example, articular cartilage normally does not undergo endochondral bone formation. However, in the osteoarthritic articular cartilage where the matrix is destroyed, chondrocytes resume proliferation and type X collagen, a hallmark of cartilage hypertrophy, is synthesized (von der Mark et al., 1992b). Thus, hypertrophy of chondrocytes is reinitiated (Hoyland et al., 1991). It is unclear what determines chondrocytes to undergo regeneration, dedifferentiation, or hypertrophy (von der Mark et al., 1992a).

There are three major types of ECM molecules in cartilage: collagens, proteoglycans, and noncollagenous glycoproteins. These molecules play an important morphogenetic role during endochondral bone formation. The expression of the genes of aggrecan core protein (Argraves et al., 1981; Goetinck, 1991) and collagens type II (Bogaert et al., 1992; Garofalo et al., 1991, 1993; Metsaranta et al., 1992), type IX (Nakata et al., 1993), and type X (Jacenko et al., 1993). As part of this study we investigated the gene expression of cartilage matrix protein (CMP) during endochondral bone formation. CMP is a major noncollagenous protein in the matrix of cartilage (Paulsson and Heinägård, 1981). It contains two homologous repeats (CMP-like domains) that are separated by an EGF-like domain (Argraves et al., 1987; Kiss et al., 1989). CMP-like domains have also been found in collagen types VI, VII, XII, and XIV; von Willebrand factor; complement factors B and C2; the alpha chains of the integrins Mac-1, p150/95, LFA-1, and VLA-2; malaria thrombospondin-related anonymous protein; dihydropyridine-sensitive calcium channel; and inter-alpha-trypsin inhibitor (Winterbottom et al., 1992; Yamagata et al., 1991; Gerecke et al., 1993; Parente et al., 1993; Bork and Rohde, 1991). In the matrix of cartilage, CMP interacts with collagen fibrils (Winterbottom et al., 1992) and proteoglycans (Paulsson and Heinägård, 1979). The collagen binding of CMP has been localized to the CMP domains. Thus, it has been suggested that CMP may be involved in collagen fibrillogenesis and in the organization of the matrix of cartilage (Tondravi et al., 1993).

The results of the present study indicate that the CMP gene is transcribed only in the postproliferative chondrocytes in a zone between the zones of proliferation and hypertrophy of the growth plate. CMP translation products are present in the matrix surrounding the nonproliferative chondrocytes of both the zones of maturation and hypertrophy. Thus, CMP is a marker for postmitotic chondrocytes. As a result of the changes in gene expression during the differentiation program, chondrocytes in each zone reside in an extracellular matrix with a unique macromolecular composition. Chondrocytes in cell culture proceed through the same temporal progression of proliferation, maturation, and hypertrophy in that CMP is produced by postproliferative chondrocytes before type X collagen is synthesized. Finally, hypertrophic chondrocytes that are released from their surrounding environment are capable of recapitulating all three stages of the differentiation program in vitro.

**MATERIALS AND METHODS**

**Cell and Organ Culture**

Primary cultures of chondrocytes were established as described by Schmid and Conrad (1982). Briefly, tibiotarsi were dissected from stage 41 (15th day of incubation) chick embryos (Hamburger and Hamilton, 1951). The bone shell and the tarsus region were separated from the cartilaginous growth plate and discarded. To ensure that the selected regions of chondrocytes were derived from the desired zone, only cells at the center of any one zone were used for cell or pellet culture. The cells at the boundary with neighboring zones were discarded. In addition, control experiments were carried out by immunostaining with mAbs, to ensure that a cartilage piece was only positive for the molecular marker that is characteristic for chondrocytes of the desired zone.

For organ culture, cartilage explants are cultured in Ham F-12 medium containing 10% fetal calf serum (plating medium) (Gibco, Grand Island, NY) at 37°C in an atmosphere of 5% CO₂. For monolayer cell culture and pellet mass
culture, a piece of cartilage from a defined zone was subjected to enzymatic treatment with 0.1% trypsin (Sigma, St. Louis, MO), 0.3% collagenase (Worthington, Freehold, NJ), and 0.1% type I testicular hyaluronidase (Sigma) (dissociation medium). After an incubation of 30 min at 37°C this dissociation medium was removed and replaced with fresh dissociation medium and incubated at 37°C for an additional hour. For monolayer cell culture, chondrocytes were resuspended in plating medium containing 0.01% testicular hyaluronidase. After culturing overnight, the medium of the chondrocytes was replaced with fresh medium without hyaluronidase. The medium was changed every other day. Pellet mass cultures were performed as described by Kato et al. (1988). Briefly, chondrocytes were washed and pelleted at 1000g in a 15-ml plastic centrifuge tube and incubated in plating medium at 37°C.

**Determination of DNA Content**

DNA content from organ or pellet mass cultures was determined as described by Labarca and Paigen (Labarca and Paigen, 1980). Briefly, a piece of cartilage or cell pellet was homogenized in phosphate-saline buffer (PSEB: 0.05 M Na2HPO4, 2 M NaCl, 0.003 M EDTA, pH 7.4), and sonicated briefly. Hoechst nuclear dye (Polysciences, Inc., Warrington, PA) was added in 1:100 dilution in PSEB and incubated at room temperature for 1 hr. Fluorescence was measured with a TKO 100 fluorometer from Hoefer (San Francisco, CA). DNA standards were used and the determinations were made in the linear range of the curve.

**In Situ Hybridization**

In situ hybridization for mRNAs encoding aggrecan core protein, link protein, CMP, and type X collagen were performed according to Hayashi et al. (1986) and Linsenmayer et al. (1991). The slides were viewed by dark-field and bright-field optics and photographed on a Microphot-FXA microscope from Nikon (Melville, NY). The probe for CMP was CMP 1 (Argraves et al., 1987). The probe for aggrecan core protein was PG 525 (Stirpe et al., 1987). The link protein probe was LPG2 (Deák et al., 1986). The type X collagen probe was a Pstl/Pvull fragment of pYN 3116 (Ninomiya et al., 1986).

**BrdU Incorporation and Detection**

Incorporation and detection of BrdU in cartilage tissues were performed with the 5-bromo-2′-deoxyuridine labeling and detection kit II from Boehringer-Mannheim (Indianapolis, IN). Labeling cartilage with BrdU was carried out in vivo or in vitro. Both methods gave identical results. For in vivo labeling, 40 μl of BrdU (1 μl/g of body weight) were injected into the aircell of an egg 3 hr before dissection. For in vitro tissue labeling, a tibiotarsus was dissected out of the embryonic chick leg. Perichondrium and bone shell were then peeled from the cartilage to ensure penetration of BrdU into the tissue. Different labeling time periods were tested. One hour was the minimum time for intensive labeling. For cell culture labeling, BrdU (1 μM) is added to cell culture 1 hr before immunostaining.

**Immunocytochemistry and Histochemistry**

Tissue embedding, processing for cryostat sectioning, and indirect immunofluorescence for immunocytochemical analyses were performed as described by Fitch et al. (1982). For double immunofluorescence staining, cultured cells were fixed at −20°C with 70% ethanol, 50 mM glycine, pH 2.0, for 20 min. Slides were then washed with phosphate-buffered saline (PBS) and incubated with primary antibodies. After washing with PBS, secondary antibodies were applied together with Hoechst nuclear dye. Slides were washed and mounted in 95% glycerol in PBS. Double or triple exposure photography was performed with a microscope from Nikon.

The monoclonal antibody against CMP, 1H1/E2, was generated as described in detail by Binette et al. (1994). 1H1/E2 (IgG1, κ) recognizes purified chick CMP (100 μg/ml) in ELISA up to a dilution of 10−3 of ascites fluid and does not recognize purified chick collagen type II or purified chick link protein. 1H1/E2 also recognizes CMP as a single band of 54 kDa (Winterbottom et al., 1992), when total proteins from cartilage extracts are electrophoresed on 8% SDS-PAGE gels under reducing conditions. The other mAbs employed recognize link protein (486/AS; Binette et al., 1994), type II collagen (II-II 6B3; Hendrix et al., 1982), and a helical determinant in type X collagen (X-AC9; Schmid and Linsenmayer, 1985). The mAb against BrdU was purchased from Boehringer-Mannheim. The polyclonal antibodies (RC1) were generated in rabbit against purified chicken CMP. They were characterized by both ELISA and Western blot analysis as recognizing CMP specifically. Affinity-purified rhodamine or fluorescein-conjugated secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA).

**Western Blot Analysis**

Chick sterna were homogenized in 4 M urea, 50 mM Tris at pH 7.5. The homogenate was centrifuged and the amount of protein in the supernatant was measured by the optical density of the sample at 280 nm. Five micrograms of each supernatant were analyzed on a discontinuous 8% SDS-PAGE. The proteins were transferred onto Immobilon-PVDF membrane (Millipore Corp., Bedford, MA) in 25 mM Tris, 192 mM glycine, 15% methanol. The membranes were blocked in 2% bovine serum albumin fraction V (Sigma) in PBS for 30 min and then probed with the anti-CMP mAb1H1/E2, or with a polyclonal Ab EM 77 against type X collagen (Pacifi et al., 1991). Horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) or anti-rabbit IgG (Bio-Rad Lab, Melville, NY) was used as a secondary antibody. Visualization of the immunoreactive proteins was
achieved using the ECL Western blotting detection reagents (Amersham Corp., Heights, IL) and exposing the membrane to Kodak X-Omat AR film.

RESULTS

The CMP Gene Is Transcribed Only by Chondrocytes of the Zone of Maturation

To identify different stages in the chondrocyte differentiation program, we used the incorporation of BrdU to visualize proliferating chondrocytes, and probes that allowed the determination of the transcriptional activity of a number of genes that encode ECM molecules. A bright-field histological section of a growth plate of a stage 41 embryo is shown in Fig. 1A. Histologically identifiable zones are indicated. Incorporation of BrdU by the nuclei identifies the zone of proliferation (P) at the distal end of the tibia (Fig. 1B). Chondrocytes in this region (zone 1) are small and round, with many mitotic figures in the nuclei (Fig. 2, 1). In the zone of hypertrophy (zone 3), the size of the cells is dramatically increased (Fig. 2, 3). This zone (H) is characterized by the presence of the mRNA for type X collagen (Fig. 1F), a hypertrophic cartilage-specific molecule (Schmid and Linsenmayer, 1983). A comparison of Figs. 1B and 1F indicates that the proliferative zone is not contiguous with the hypertrophic zone. Therefore, there exists an additional zone between the zones of proliferation and hypertrophy. This zone corresponds to the histologically identified zone of maturation (Kim and Conrad, 1977; Stocum et al., 1979) and the CMP gene is transcribed only by chondrocytes in this zone (M) (Fig. 1C). Flattened cells at the beginning of this zone (2a) give rise to medium-sized cells toward the end of the zone (2b), fulfilling the transition from the proliferating to the hypertrophic state (see Figs. 1A and 2, 2a and 2b). Transcripts for aggrecan core protein and link protein are present in chondrocytes of both the proliferative zone and the zone of maturation, but not in those of the zone of hypertrophy (Figs. 1D and 1E). Thus, the spatially restricted transcriptional activity of the CMP gene in the growth plate indicates that the chondrocytes situated between the chondrocytes that make up the zones of proliferation and hypertrophy are a unique population of cells. These observations clearly establish the presence of at least three stages in the differentiation program of chondrocytes.

The Translation Product of the CMP Gene Is a Marker for Postproliferative Chondrocytes

The distribution of ECM proteins and incorporated BrdU was examined in sections from the tibiotarsus of a stage 45 chick embryo. BrdU labeling (Fig. 3A) revealed a zone of proliferating chondrocytes in the epiphyseal growth plate. At this stage the proliferative zone is shorter than the one at stage 41 (compare Figs. 3A and 1B). There are many proliferating chondrocytes at the perimeter of the growth plate, reflecting the rapid appositional growth of the rudiment (Fig. 3A). Proliferating cells of the blood or bone marrow could also be identified (Fig. 3A, white arrows). CMP could be shown by immunofluorescence to be present in the two postproliferative zones of maturation and hypertrophy as well as in the tarsus (Fig. 3C). CMP is absent not only from the proliferative zone, but also from the proliferating chondrocytes at the perimeter of the tibia (compare Figs. 3C and 3A). Type X collagen was present only in the hypertrophic zone (Fig. 3D). In contrast to the specific localization of

FIG. 1. Micrographs of sections of a tibiotarsus from a stage 41 chick embryo. Bright-field histochemical staining (H&E) (A), immunofluorescence micrograph with a mAb against incorporated BrdU (B), and dark-field micrographs of in situ hybridizations with cDNAs for CMP (C), the core protein of aggrecan (D), link protein (E), and type X collagen (F). P and 1, zone of proliferation (zone 1); M and 2, zone of maturation (zone 2); 2a, first half of the mature zone; 2b, second half of the mature zone; H and 3, zone of hypertrophy (zone 3). Bar, 400 μm.
FIG. 2. Morphology of chondrocytes during in vivo differentiation in the growth plate of a stage 41 embryonic chick tibiotarsus (bottom) compared with the changes of the proliferative zone explants incubated in culture (top). The sections are from the proliferative zone (1), first half of the mature zone (2a), second half of the mature zone (2b), and the hypertrophic zone (3) in vivo or the explant from the proliferative zone incubated in culture for 1 hr (1h), 20 hr (1d), and 5 days (5d). All the sections are stained with hematoxylin and eosin. Arrows in (1) indicate dividing chondrocytes. Bar, 10 μm.

CMP and type X collagen, link protein was detected throughout the growth plate (Fig. 3B). Both CMP and link protein were present in the matrix surrounding chondrocytes of the hypertrophic region even though the genes for these proteins are not transcribed by the chondrocytes of that zone (Figs. 3C and 3B). Thus, the translation product of the CMP gene is a marker for nonproliferating chondrocytes of either the mature or the hypertrophic zone. These results indicate that the synthetic repertoire of chondrocytes changes as differentiation proceeds and, as a result, the composition of matrix is modified during the progression of the differentiation program.

Spatial and Temporal Expression of the Differentiation Program in Primary Chondrocyte Cultures

To test whether the spatial organization of chondrocytes into distinct zones in vivo reflects the manifestation of their differentiation process temporally, we examined the differentiation time sequence of chondrocytes in monolayer cell culture. Chondrocytes were isolated from the core of the proliferative zone of tibiae from stage 41 chick embryos and cultured. After 3 days of culture, CMP can be detected in the cultures, but only in a subset of the chondrocytes. The

FIG. 3. Fluorescence micrographs of sections from a tibiotarsus of a stage 45 chicken embryo. Reactions with monoclonal antibodies against incorporated BrdU (A), link protein (B), CMP (C), and type X collagen (D) are shown. P, zone of proliferation; M, zone of maturation; H, zone of hypertrophy. White arrows point to the proliferation of cells from blood or bone marrow. Bar, 300 μm.
FIG. 4. Fluorescence staining of primary cultures of chondrocytes isolated from the proliferative zone of the epiphyseal growth plate. The presence of the cells are indicated by the blue nuclei staining with the Hoechst dye. (A, B, and C) From 3-day cultures. (D) is from a 15-day culture. (A) Cells stained with mAb 1H1/E2 against CMP (red). Notice the red staining only in the center of the colonies. (B) Cells doubly stained with mAb II-II 6B3 against type II collagen (red) and RC-1 against CMP (green). The solid arrow points to the type II collagen staining in the cells. The open arrow points to the CMP staining. The overlapping region of type II (red) and CMP (green) is yellow, which is only at the center of the colony. (C) Cells doubly stained with anti-BrdU (red) and RC1 against CMP (green). The solid arrow points to the BrdU staining in the nuclei of the cells at the periphery of the colony, suggesting that they are proliferating cells. The open arrow points to the blue nuclei (BrdU negative) in the center of the colony. These cells are CMP positive (green). (D) Chondrocytes after 15 days of culture. Only part of a colony is shown. Cells doubly stained with RCI against CMP (green) and mAb X-AC9 against type X collagen (red). The solid arrow points to the filamentous staining of CMP connecting the cells in the colony. The open arrow points to the punctate staining of type X collagen in the center of the colony. Bar, 225 nm.

CMP-positive cells are located at the center of the colonies (Fig. 4A). The cells at the periphery of the colonies do not stain for CMP. The CMP distribution pattern is in contrast to that of type II collagen, which can be detected in the cells throughout the colonies (Fig. 4B). To test whether the CMP-negative cells at the periphery are in fact the proliferating chondrocytes, the cultures were incubated in the presence of BrdU for 1 hr. The peripheral cells which lack CMP actively incorporate BrdU (Fig. 4C), indicating that they are proliferating chondrocytes. BrdU incorporation cannot be detected in the chondrocytes at the center of the colonies which produce CMP (Fig. 4C), indicating that these are post-proliferative cells. After 15 days of incubation, chondrocytes have stopped dividing, as indicated by the lack of BrdU incorporation (data not shown). CMP is laid down in the extracellular space as filamentous material throughout the colonies (Fig. 4D). At this time, type X collagen can be detected as punctate fluorescence signals within the colonies. Thus, in the culture system, the chondrocytes go through the same differentiation program as they do in vivo. A wave of differentiation spreads from the center of the colonies to the periphery as proliferative cells differentiate into mature and subsequently into hypertrophic chondrocytes.

Removal of the Extracellular Environment from Proliferative Chondrocytes Prolongs Their Proliferation and Delays Their Maturation

The data presented above suggest that there is a correlation between the state of differentiation of chondrocytes...
FIG. 5. Diagram describing the method used to generate differentiation stage-specific organ cultures and pellet mass cultures. P, proliferative zone; M, mature zone; H, hypertrophic zone. These three zones were separated by microdissection. The cartilage explants were either directly incubated as organ cultures or digested with collagenase, testicular hyaluronidase, and trypsin to eliminate the extracellular environment. These chondrocytes were washed and pelleted and incubated as pellet cell cultures. The proliferative zone and the hypertrophic zone were tested in this experiment.

and their ECM expression. To test if this state is directly influenced by the extracellular environment surrounding differentiating chondrocytes, an in vitro culture system was devised in which pieces of cartilage are taken from the core of the zones of proliferation and hypertrophy (Fig. 5). In one set of experiments the chondrocytes are maintained as an isolated piece of cartilage surrounded by their original extracellular matrix (organ culture). These are compared with the chondrocytes that are enzymatically removed from their surrounding matrix and cultured as a pellet mass culture (pellet culture). The organ and pellet cultures were carried out with chondrocytes that originated from the same region of the growth plate. For each comparison, therefore, the chondrocytes are from the same developmental stage.

When a piece of cartilage from the proliferative zone is maintained as an organ culture in the presence of serum the DNA content remains constant during 12 days of incubation (Fig. 6, solid squares). This observation indicates that the chondrocytes have stopped proliferating in culture. The morphology of the chondrocytes of the cartilaginous pieces cultured for 1 hr, 1 day, or 5 days is very similar to that seen in the mature (zones 2a and 2b) and the hypertrophic (zone 3) zones, respectively (compare Fig. 2, 1h to 2a, 1d to 2b, and 5d to 3). These changes in morphology suggest that the chondrocytes have undergone differentiation in culture.

This rapid process of differentiation in vitro from the proliferative through the mature to the hypertrophic stage was demonstrated at the molecular level by the incorporation of BrdU (marker for proliferation) and the expression of CMP (marker for maturation) and type X collagen (marker for hypertrophy) (Fig. 7). The incorporation of BrdU by the cells during the first hour of incubation confirms their identity as proliferating chondrocytes (Fig. 7A). There is little staining for the postmitotic chondrocyte marker CMP at the end of the first hour (Fig. 7B), and there is no staining for type X collagen (data not shown). However, after 1 day of incubation (20 hr), there is strong staining for CMP (Fig. 7D), no detectable BrdU incorporation (Fig. 7C), and still no staining for type X collagen (data not shown). These changes suggest that the chondrocytes have differentiated to the stage of maturation. Type X collagen can be first detected at the 3rd day of incubation (data not shown). After 5 days of incubation, type X collagen can be detected throughout the cultured piece (Fig. 7F) and there is no BrdU incorporation detected (Fig. 7E), indicating that the chondrocytes have reached the hypertrophic stage. The results also suggest that the constant level of DNA in the cultured pieces of cartilage is the result of the maintenance of living cells and not the result of a balance between proliferation and cell death. In contrast, in organ cultures without serum, DNA content in the cultured pieces decreases by 50% during incubation (Fig. 6, solid diamonds). However, in these cultures the chondrocytes go through the same differentiation sequence as the cells cultured in the presence of serum (data not shown).

Therefore, in organ culture, serum is necessary for preventing cell death or tissue degradation, but not required for chondrocyte differentiation.

To compare quantitatively the appearance of CMP and type X collagen during incubation of the cartilage explants from the proliferative zone, an equal amount of extracts (5 μg) from cartilage explants cultured for 0, 1, and 5 days are analyzed by Western blot. As shown in Fig. 7G, a band of CMP trimers (200 kDa) is barely detectable at the beginning of incubation (0 day). It is clearly detected after 1 day of
incubation and is present at the 5th day of incubation. In comparison, type X collagen (59 kDa) is not detected at either 0 or 1 day of incubation, but is detected at the 5th day of incubation. Thus, when chondrocytes from the proliferative zone are cultured as an organ, proliferation ceases and differentiation starts during the 1st day of incubation. In contrast, in pellet mass cultures of proliferative chondrocytes that are removed from their extracellular environment, proliferation continues for 10 days (Fig. 6, open square). These chondrocytes synthesize a new surrounding matrix (data not shown) before undergoing subsequent maturation and hypertrophy. Therefore, the transient removal of the extracellular environment from proliferating cells in situ prolongs the proliferative period and delays the progression of the differentiation program.

**Hypertrophic Chondrocytes Can Recapitulate the Chondrocyte Differentiation Program**

The behavior of postmitotic, "terminally" differentiated hypertrophic chondrocytes was examined in organ and in pellet mass cultures. Pieces of hypertrophic cartilage were taken from the core of late hypertrophic zones. Chondrocytes in these pieces are large in size (see Fig. 2) and they actively transcribe the type X collagen gene (Fig. 9C). To eliminate the complication of cell death, hypertrophic chondrocytes were incubated only in serum containing medium. In organ culture, the DNA content of the incubated pieces of hypertrophic cartilage remains constant (Fig. 8, solid squares). However, in pellet mass cultures, the postmitotic hypertrophic chondrocytes that were deprived of their extracellular surrounding in situ resume proliferation and double their DNA content by 6 days (Fig. 8, open squares). To determine if the resumption of proliferation represents the beginning of the recapitulation of the differentiation program, we examined, by in situ hybridization, the expression of the genes encoding aggregan core protein and CMP during the incubation of the chondrocytes in pellet cultures. These two genes are not active in hypertrophic chondrocytes (Figs. 9A and 9B). At Day 4 of incubation, the transcription of the genes for aggregan core protein and CMP is reinitiated throughout the pellet mass culture derived from the hypertrophic zone (Figs. 9D and 9E). The presence of transcripts for aggregan core protein is still present at Day 7 (Fig. 9G) and the transcription of CMP seems to reach a peak at this time. Both the density and intensity of the grains for CMP (hybridization signals) increase (Fig. 9H). This suggests that there is an elevation in the transcriptional activity of the CMP gene by an increased number of cells. There is no longer any increase in DNA content (Fig. 8, open squares) at this time. In contrast, the transcription of the type X collagen gene, a marker for hypertrophic cartilage (Fig. 9C), is greatly down-regulated at 4 days of incubation (Fig. 9F). At Day 12 of incubation, the expression for type X collagen is reinitiated (Fig. 9I). The histochemical staining of a Day 12 pellet mass culture shows the de novo synthesized matrix material that is deposited between the cells by the chondrocytes during the incubation period (Fig. 9, arrow).

The sections of the pellet cultures and organ cultures of hypertrophic chondrocytes were examined by immuno-staining with mAbs against incorporated BrdU and type X collagen. At Day 4 of incubation, there are many BrdU-positive nuclei in the pellet culture (Fig. 10A) and none in the organ culture (Fig. 10C). However, at Day 7 of incubation, there are only a few BrdU-positive nuclei in the pellet culture (Fig. 10E) and still none in the organ culture (Fig. 10G). These results confirm the measurement of DNA content in both pellet and organ cultures (Fig. 8). Furthermore, type X collagen is distributed throughout both pellet and
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content and incorporation of BrdU), maturation (CMP expression), and hypertrophy (type X expression).

DISCUSSION

The Cellular Organization of the Growth Plate

The chondrocytes of the growth plate progress through a differentiation program which can be partitioned into a number of stages. These stages are clearly reflected in the spatial organization of the chondrocytes in the different zones of the growth plate. The generally accepted model, as presented in many textbooks (Ross and Romrell, 1989; Gilbert, 1994), describes the proliferative zone in a growth plate as immediately preceding the hypertrophic zone. Our study clearly shows that, at least in chick embryos, this is not the case. The distribution of proliferating chondrocytes in a growth plate was determined by monitoring the localization of BrdU incorporated by dividing chondrocytes at the G1-S phase transition. The zone of hypertrophic chondrocytes was identified by the expression of type X collagen, a commonly accepted marker of hypertrophic chondrocytes (Schmid and Linsenmayer, 1983). Our results clearly indicate that the proliferating chondrocytes make up a zone which is not contiguous with the hypertrophic zone. Therefore, there exists a population of nonproliferating chondrocytes that are not yet hypertrophic. Here we identify the transcription of the CMP gene as a molecular marker for chondrocytes of that zone. Our observations are consistent with a second model which proposes that the chondrocytes of a growth plate are divided into at least three distinct stages that have been identified histologically as the zones of proliferation, maturation, and hypertrophy (Stocum et al., 1979; Oohira et al., 1974). The zones that we identify...
ther by determining the pattern of transcription of genes that encode chondrocyte matrix molecules. Our report is the first to identify CMP as a product of and a marker for mature chondrocytes. A restricted pattern of expression of the CMP gene in the growth plate has also been reported in human and mouse (Aszódi et al., 1994; Mundlos and Zabel, 1994). However, in both reports it is assumed that the proliferative zone is contiguous with the hypertrophic zone, and the zone of proliferation was not identified experimentally in either study. In human embryonic long bones the mRNA for CMP was reported to be present in the upper "hypertrophic" and lower "proliferative" zone (Mundlos and Zabel, 1994). This region is likely to correspond with the zone of maturation as defined in the present study. In another report (Aszódi et al., 1994) the expression of a chicken genomic clone was monitored by immunohistochemical means in transgenic mice. High levels of chicken CMP were reported to be present in the zone of proliferating chondrocytes and not in the zone of hypertrophic chondrocytes. It is difficult to compare the interpretation of these results with those of the present study since the proliferating chondrocytes were not identified in the transgenic animals. The reported absence of the translation product of the chicken CMP gene in the hypertrophic zone of the transgenic mice differs from our observation (Chen et al., unpublished) that mouse CMP is present in the hypertrophic region of normal mice.

It has been reported that CMP is not detected in the superficial layer of chondrocytes covering the joint articular surface (Franzen et al., 1987; Mundlos and Zabel, 1994), but is detected in the deep layer of articular cartilage (Mundlos and Zabel, 1994). This observation is reminiscent of the distribution pattern of CMP in the embryonic growth plate described here. Therefore, we suggest that the difference in CMP distribution is due to the different stages of cartilage development, rather than the different types of cartilage. Consistent with this idea are our previous results (Stirpe and Goetinck, 1989), which indicated that the transcripts for CMP are first detected at stage 26 during chick limb development, later than those for type II collagen (stage 23 and those for link protein and aggrecan core protein (stage 25).

The Differentiation Program of Chondrocytes

The results of the analysis of the spatial arrangement of chondrocytes in the growth plate is corroborated by the study of chondrocytes in monolayer cell culture in which differentiation events happen sequentially. The analysis in vitro offered an advantage since immunostaining of chondrocytes in culture allows the characterization of differentiation events in individual cells. Chondrocytes isolated from the proliferative zone of the epiphyseal growth plate go through the stages of proliferation, maturation, and hypertrophy in vitro as they do in vivo. It is demonstrated by our cell culture experiment that CMP is only made by those cells that are no longer proliferating (lack of BrdU incorporation.)
FIG. 10. Immunofluorescence micrographs of sections from organ cultures or pellet mass cultures of hypertrophic chondrocytes. (A and B) From 4-day pellet cultures; (C and D) from 4-day organ cultures; (E and F) from 7-day pellet cultures; (G and H) from 7-day organ cultures. (A, C, E, and G) Reacted with a mAb against BrdU; (B, D, F, and H) reacted with X-AC9, a mAb against type X collagen. Bar, 50 μm.

Therefore, CMP contributes to the formation of the cartilage matrix established by postproliferative chondrocytes, before the matrix is further modified by type X collagen synthesized by hypertrophic chondrocytes. These results indicate that the properties of the cartilage matrix change during the course of differentiation. Furthermore, this modification of the matrix is controlled, at least in part, at the mRNA level. This is illustrated by the in situ hybridization data of the exclusive synthesis of mRNA for CMP by mature chondrocytes and of mRNA for type X by hypertrophic chondrocytes.

The program of chondrocyte differentiation is summarized in Fig. 11. When chondrogenesis is initiated, the genes for collagens type II (Miller, 1972) and IX (Linsenmayer et al., 1991), aggrecan core protein, and link protein (Figs. 1D and IE) are activated and the gene for type I collagen is repressed (Linsenmayer et al., 1973). The switch from mesodermal precursor cells to proliferating chondrocytes is the first step in the cartilage differentiation program. The gene for CMP is activated when chondrocytes stop proliferating and undergo maturation. This step represents the second switch in the program. When chondrocytes undergo hypertrophy, the genes that encode CMP, link protein, and aggrecan core protein are repressed and the gene for type X collagen is activated. This represents the third switch in the program. These three switches represent key transition points in chondrogenesis and endochondral bone formation. The first switch separates chondrocytes from their mesodermal precursors and the second switch separates proliferating chondrocytes from postmitotic mature chondrocytes. In the “permanent” cartilage such as articular cartilage and the cartilage of the caudal portion of the sternum, chondrocyte differentiation stops at the maturation stage. If the third switch is activated, chondrocytes proceed to the hypertrophic stage. Therefore, this switch separates the permanent cartilage from the transitional cartilage which serves as a model for endochondral bone formation.

The Chondrocyte Differentiation Program Is Reversible

In the present study we also show that the chondrocyte differentiation program is dynamic. It can be delayed, re-
versed, and recapitulated. Our data suggest that the surrounding extracellular environment is essential for maintaining the state of differentiation of chondrocytes. The extracellular cues from this microenvironment may include both ECM molecules and growth factors. It has been shown that growth factors such as TGF-β and b-FGF are important in modulating and preventing chondrocyte hypertrophy during endochondral bone formation (Ballock et al., 1993; Kato et al., 1988). Furthermore, growth factors have been shown to bind to the extracellular matrix network. Thus, the transient removal of the surrounding matrix may also affect the localized activities of growth factors. Alternatively, the reinitiation of chondrocyte proliferation and the temporary loss of the differentiated phenotype could result from an alteration of cell–matrix interactions originally present in cartilage and maintained by the matrix architecture. This hypothesis is also consistent with our data which indicate that, when matrix is resynthesized and deposited following initial cell isolation and incubation in pellet culture, chondrocyte proliferation ceases and differentiation proceeds.

Finally, we have demonstrated that the activation and repression of genes during endochondral bone formation are reversible events and that terminally differentiated chondrocytes can reverse their differentiated state and recapitulate the entire differentiation program. The mechanism for the retrodifferentiation from postmitotic, differentiated hypertrophic chondrocytes to proliferating chondrocytes is different from the dedifferentiation of proliferating chondrocytes to fibroblast-like cells (Fig. 11). Retrodifferentiation involves the switch between proliferation and differentiation within the chondrocyte differentiation program, whereas dedifferentiation involves changes to a nonchondrogenic phenotype. Our observations suggest that the terminally differentiated chondrocytes are developmentally flexible in that they still have the potential to regenerate ECM and form a “new” cartilage. As mentioned above, we are the first to report the reversal of differentiation from terminally differentiated chondrocytes, similar examples have been reported in muscle cells during newt limb regeneration (Lo et al., 1993). The retrodifferentiation from postmitotic myoblasts observed to myoblasts is shown to be mediated by retinoblastoma protein (Rb) (Gu et al., 1993) or p107 in the Rb-deficient cell lines (Schneider et al., 1994). The regulatory pathway of chondrocyte differentiation remains to be fully explored and understood. However, this report, together with other previous studies (Cancedda et al., 1992; Bruckner et al., 1989; Schmid et al., 1990), provides a basis for elucidating the complicated and yet well-regulated mechanisms of chondrocyte differentiation during bone development and repair.

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