

Gene Expression during Osteogenic Differentiation in Mandibular Condyles In Vitro

P. Günter Strauss, Ellen I. Closs, Jörg Schmidt, and Volker Erfle

GSF-Abteilung für Molekulare Zellpathologie, D-8042 Neuherberg, Federal Republic of Germany

Abstract. The cartilagenous tissue of mandibular condyles of newborn mice contains progenitor cells as well as young and mature chondrogenic cells. During in vitro cultivation of the tissue, progenitor cells undergo osteogenic differentiation and form new bone (Silbermann, M., D. Lewinson, H. Gonen, M. A. Lizarbe, and K. von der Mark. 1983. *Anat. Rec.* 206:373–383). We have studied the expression of genes that typify osteogenic differentiation in mandibular condyles during in vitro cultivation. RNAs of the

genes for collagen type I, osteonectin, alkaline phosphatase, and bone gla protein were sequentially expressed in progenitor cells and hypertrophic chondrocytes during culture. Osteopontin expression peaked in both the early and the late phase of the differentiation process. The data indicate a distinct sequence of expression of osteoblast-specific genes during osteogenic differentiation and new bone formation in mandibular condyles.

BONE- and cartilage-forming cells are believed to be derived from a common progenitor cell (for review see Marks and Popoff, 1988). Whereas various authors have indicated the possibility of an interconvertibility between the osteogenic and chondrogenic cell lineages (Knese and Knoop, 1961; Holtrop, 1966; Crelin and Koch, 1967; Kuhlman and McNamee, 1970; Lutfi, 1971; Silbermann and Frommer, 1972, 1974; Shimomura et al., 1973; Kahn and Simmons, 1977; Yoshioka and Yagi, 1988), others have disputed this concept (Caplan et al., 1983). It has long been thought that after maturation the hypertrophic chondrocytes degenerate and die. This hypothesis, however, has recently been challenged, and it has been suggested that differentiated cartilage cells may be actively involved in bone formation (for review see Cowell et al., 1987; Yoshioka et al., 1988).

The cartilagenous tissue of the mouse mandibular condyle cultured in vitro (Silbermann et al., 1986) represents a powerful model to study questions related to these aspects of skeletoblast differentiation. The system has been used to study the effect of external factors regulating cell growth and differentiation on bone formation (Silbermann et al., 1983; Silbermann and Maor, 1985; Lewinson and Silbermann, 1986), to induce osteosarcoma-like lesions with the Finkel Biskis Reilly murine osteosarcoma virus (Schmidt et al., 1986, 1989a,b; Silbermann et al., 1987) and to investigate the effects of bone-pathogenic retroviruses on condylar tissue in vitro (Livne et al., 1989; Schmidt et al., 1989b). The mandibular condyle is composed of distinct zones containing cartilage cells at different stages of differentiation. The tissue is surrounded by mesenchyme-like cells of the perichondrium. A zone of progenitor cells composing an apical and lateral sheath encompasses an area of young chondro-

blasts in the center and hypertrophic chondrocytes in the basal part. During in vitro cultivation, cells in the chondroprogenitor zone undergo osteogenic differentiation and form new bone (Silbermann et al., 1983).

Osteogenic differentiation in vivo is accompanied by the expression of characteristic genes, including collagen type I, alkaline phosphatase, osteonectin, osteopontin, and bone gla protein (BGP, osteocalcin).¹ Whereas BGP, a specific marker for osteogenic cells, is exclusively expressed in the late phase of bone cell differentiation (Celeste et al., 1986; Yoon et al., 1988; Lian et al., 1989), collagen type I, the major constituent of bone matrix, is also expressed in a variety of other cell types (Kühn, 1986) including chondroprogenitor cells (von der Mark, 1980; Castagnola et al., 1988), as are alkaline phosphatase, a hallmark molecule for bone-forming cells, osteonectin, and osteopontin (Terao and Mintz, 1987; Murthy et al., 1986; Mason et al., 1986; Stenner et al., 1986; Yoon et al., 1987; Nomura et al., 1988). Nevertheless, osteogenic differentiation of skeletal cells is characterized by joint expression of these proteins.

We have investigated the expression of these marker genes in mandibular condyles in culture. RNA from the individual genes was expressed in a temporal pattern, indicating progressing states of osteogenic differentiation of progenitor cells and hypertrophic chondrocytes to osteoblast-like cells.

Materials and Methods

Organ Cultures

Mandibular condyles were obtained from mandibles of newborn National Medical Research Institute mice (from the breeding colony of the growth

1. *Abbreviation used in this paper:* BGP, bone gla protein.

stimulating factor [GSF]). The explants were freed from adherent tissue and mandibular bone, transferred onto collagen sponges (Colla-Tec, Plainsboro, NJ) and cultured as described (Schmidt et al., 1986). Fresh specimens were obtained directly after dissection and washed with ice-cold PBS. In vitro-cultured specimens, together with the collagen sponge, were removed from the medium at the time points given in Results and then washed with ice-cold PBS.

For RNA preparation the condyles were removed immediately from the collagen sponges, frozen in liquid nitrogen, and stored at -120°C .

For in situ hybridization, condyles, including the underlying collagen sponge, were immediately after removal from the medium placed in a drop of embedding medium (Reichert und Jung, Nussloch, FRG), frozen in liquid nitrogen, and stored at -80°C .

Morphology

After washing with ice-cold PBS, the condyles were fixed in 3% glutaraldehyde in cacodylate buffer, postfixed in chrome-osmium, dehydrated in alcohol, and embedded in Epon according to standard procedures. One-micrometer sections were counterstained with toluidine blue.

DNA Probes

The following DNA probes were used: Hf677 (Chu et al., 1982) for collagen type I. Hf677 is a cDNA clone derived from the human pro $\alpha 1(\text{I})$ collagen. The 1.8-kb Eco RI insert spans from the amino acid residue 787 to 270 nucleotides into the 3' end untranslated region of the pro $\alpha 1(\text{I})$ mRNA. pH CAR I (Sandberg and Vuorio, 1987) was used for collagen type II. pH CAR I was cloned from a human pro $\alpha 1(\text{II})$ collagen RNA. The clone was digested with Pvu II and Eco RI to give a 224-bp probe, specific for the carboxy-terminal propeptide region. pC33.1 (Holland et al., 1987) was used for mouse osteonectin. pC33.1 is a genomic clone of the SPARC gene. The 1.1-kb insert was released by digestion with Eco RI and Hind III. pSPmpl (Terao et al., 1987) was used for mouse alkaline phosphatase. pSPmpl is derived from the cDNA clone *lmpA*, specific for mouse placental alkaline phosphatase. A 2-kb insert was prepared from pSPmpl by digestion with Eco RI and Hind III. pBS-ROP (Yoon et al., 1987) was used for osteopontin. pBS-ROP is a cDNA clone of rat osteopontin. A 1.3-kb, osteopontin-specific insert was prepared by Eco RI digestion. p923 (Celeste et al., 1986) was used for BGP. p923 contains cDNA from mouse BGP. An 0.5-kb insert was prepared by digestion with Eco RI and Pst I. p λ Hac-69 (Moos and Gallwitz, 1983) was used for actin. p λ HAC-69 is derived from the genomic clone λ HAC-69, containing the human β -actin-like pseudogenes H β Ac- $\psi 2$. The insert was released by digestion with Hind III. Only the probe-specific inserts of the plasmids were used for the hybridizations. Inserts were ^{32}P -labeled to a specific activity of 5×10^8 cpm/ μg DNA using a random-primer labeling kit (Amersham Buchler, Braunschweig, FRG). For in situ hybridizations, the length of the labeled probes was determined on alkaline agarose gel electrophoresis and the average size adjusted to ~ 500 bp by radiolytic degradation if necessary.

Preparation of RNA

Frozen condyles from the same time points were pooled and homogenized in the presence of guanidium isothiocyanate buffer (Chirgwin et al., 1979) in liquid nitrogen. Total RNA was then prepared by ultracentrifugation of the thawed homogenate through a CsCl cushion (Chirgwin et al., 1979). The yield of total RNA from one condyle was 0.5–1.5 μg .

Northern Analysis

Electrophoresis of RNA was performed using the glyoxal method described by Maniatis et al. (1982). 5 μg of total RNA was used per lane. The RNA was transferred to ZETA-PROBE (Bio-Rad Laboratories, Inc., Brussels, Belgium) blotting membranes. The filters were hybridized at 65°C in hybridization solution (0.5 M NaH_2PO_4 , pH 7.0, 7% SDS) containing 2×10^6 cpm/ml of the labeled probe. After hybridization for 18 h, the filters were washed for 15 min at room temperature and 10 min at 65°C in 50 mM NaH_2PO_4 , pH 6.5, 1% SDS. Autoradiography was carried out at -70°C using Kodak XAR film (Eastman Kodak Co., Rochester, NY) and intensifier screens (Curix MR 800; Agfa).

In Situ Hybridizations

In situ hybridization was carried out by a modification of the procedures described by Hafen et al. (1983), Wolf et al. (1984), and Deschepper et al.

(1986). 8- μm sections were cut on a cryostat at -20°C and spread on pretreated slides (Brahic and Haase, 1978). The sections were dried on a hot plate for 5 min at 50°C , fixed in freshly prepared 4% formaldehyde (in 0.1 M phosphate buffer) for 30 min at room temperature, washed in PBS and deionized H_2O , dehydrated in ethanol, air dried, and stored at -80°C . Before hybridization, the sections were incubated in 0.2 M HCl at room temperature for 20 min, incubated in $20 \times \text{SSC}$ (0.3 M NaCl, 0.03 M sodium citrate) for 30 min at 70°C , and treated with 1 mg/ml of proteinase K for 15 min at 37°C . The sections were fixed again in formaldehyde, dehydrated in ethanol and air dried. Prehybridization was performed at 45°C for 3–4 h with 10 ml of 50% formamide, 0.6 M NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mg/ml tRNA, 0.1 mg/ml polyadenylate, 1 mg/ml salmon sperm DNA, and Denhard's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.1% BSA). Hybridization was performed in 10 μl of the same solution containing 1×10^6 cpm of ^{32}P -labeled DNA (specific activity 5×10^8 cpm/ μg DNA, DNA denatured by boiling for 10 min and chilled on ice) at 45°C for 24 h. After hybridization the slides were washed three times for 5 h each in formamide buffer (50% formamide, 0.6 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) at room temperature followed by washing in $2 \times \text{SSC}$ and in $0.2 \times \text{SSC}$ for 30 min each at 45°C . The slides were rinsed with ethanol ammonium acetate (70–90%/0.3 M), and air dried. Autoradiography was performed by dipping the slides in Kodak NTB-2 emulsion (Eastman Kodak Co.) diluted 1:2 with distilled water at 45°C . The slides were air dried at room temperature for 30 minutes and exposed at 4°C in a dry chamber. Slides were developed with Kodak D-19 developer (Eastman Kodak Co.) for 8 min, rinsed briefly with 3% acetic acid and fixed in Tetanal Superfix (Tetanal, Norderstedt, FRG) for 20 min. Afterwards the slides were counterstained with haematoxylin eosin.

Slides showing weaker hybridization signals were photographed with dark-field illumination, slides containing strong signals with bright-field illumination.

Results

I. Morphology of Mandibular Condyles

The mandibular condyle of newborn mice is composed of cartilagenous tissue at various stages of differentiation. Fig. 1 *a* shows the typical appearance of a condyle before cultivation. The perichondreal sheath encompassing the lateral and apical part of the tissue is characterized by mesenchyme-like cells. These cells serve as the source of chondroprogenitor cells in vivo. During cultivation in vitro, cells from the progenitor zone serve as a source of bone precursor cells (Silbermann et al., 1983). Progenitor cells are localized in a defined area below the perichondreal cell layer. The zone of progenitor cells is particularly large in the apical part and relatively narrow in the lateral parts of the tissue. The core of the mandibular condyle contains young chondroblasts, mature chondroblasts and hypertrophic chondrocytes at the basal area (Fig. 1).

After 7 d in culture, new bone formation was observed preferentially in the zone of progenitor cells located in the apical part. The cells in the chondroblastic zone had differentiated further towards hypertrophic chondrocytes (Fig. 1, *b* and *d*). In the hypertrophic zone we observed cells with a heterogenous, atypical morphology. These cells had lost their rounded appearance, and occasionally two or more cells were found in single lacunae (Fig. 1 *e*). Numerous osteoblast-like cells had invaded the underlying collagen sponge and formed ectopic nodules of new osteoid in the collagenous meshes (Fig. 1 *f*).

Advanced stages of osteogenic differentiation were observed by day 14 of culture (Fig. 1 *c*). The apical and lateral parts of the tissue consisted of newly formed osteoid, showing increased mineralization along the border of the progenitor cells. At this stage the progenitor cells appeared as a narrow band nearly enclosing the entire tissue. The zone of hyper-

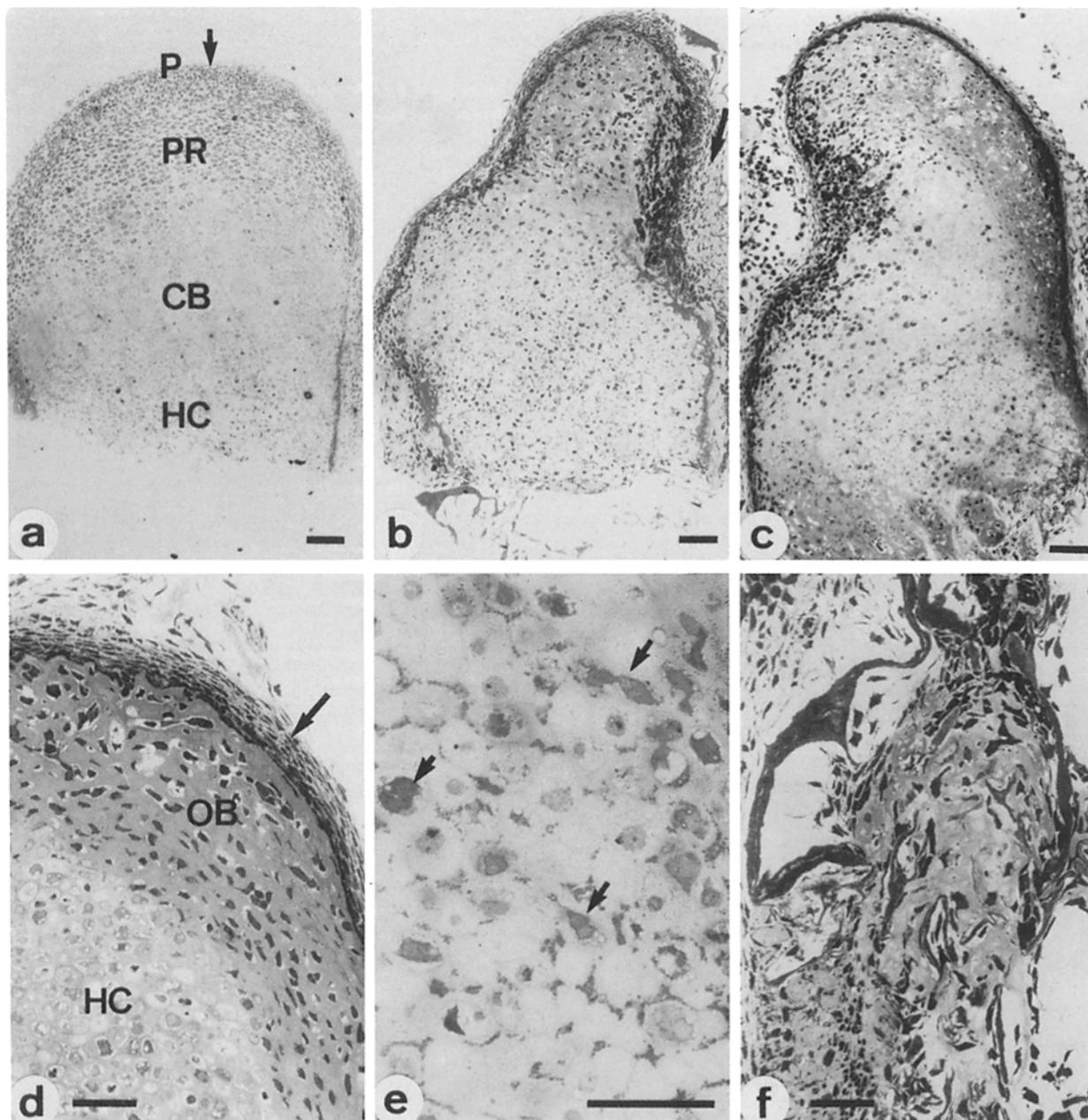


Figure 1. Morphology of mandibular condyles. (a) Fresh explant. Note the distinct layer of mesenchyme-like perichondrium cells (*P*) encompassing the apical (*arrow*) and lateral part of the tissue. The cells in the progenitor zone (*PR*) differentiate *in vitro* towards osteoblast-like cells. Mature chondroblasts (*CB*) and hypertrophic chondrocytes (*HC*) are localized in the core and in the basal part of the tissue. Bar, 100 μm . (b) Explant cultured for 7 d. Note the advanced stage of osteogenic differentiation in the area of the progenitor cells. Osteoblast-like cells invade the underlying collagen sponge (*arrow*). Bar, 100 μm . (c) Explant cultured for 14 d. Advanced osteogenic differentiation and substantial osteoid production throughout the condylar tissue encircling an area of hypertrophic chondrocytes. Bar, 100 μm . (d) Higher magnification of the apical part of a 7-d explant in culture. Osteoblasts (*OB*) in newly formed osteoid surrounded by a layer of periosteal cells (*arrow*). The core of the tissue consists of hypertrophic chondrocytes (*HC*). Bar, 50 μm . (e) Higher magnification of the basolateral part of a 7-d explant in culture. Note the appearance of two cells in some of the lacunae in the area of hypertrophic chondrocytes (*arrows*). Bar, 50 μm . (f) Osteoblast-like cells forming ectopic nodules of osteoid in the meshes of the collagen sponge. Bar, 50 μm .

trophic chondrocytes was encircled by the newly formed osteoid.

II. Expression of Bone Cell Characteristic Genes in Condyles during Culture (Northern Analysis)

The morphological evidence showing osteogenic differentiation of progenitor cells *in vitro* was further substantiated by

Northern blot analysis of the expression of genes that are usually transcribed during osteogenic differentiation (Fig. 2).

All the bone cell characteristic genes we tested showed enhanced RNA levels during culture. Collagen type I, osteonectin, alkaline phosphatase, and osteopontin were already expressed in freshly obtained condyles, but the RNA levels of these genes *in vitro* were significantly higher. The amount of collagen type I RNA increased twofold after 3 d

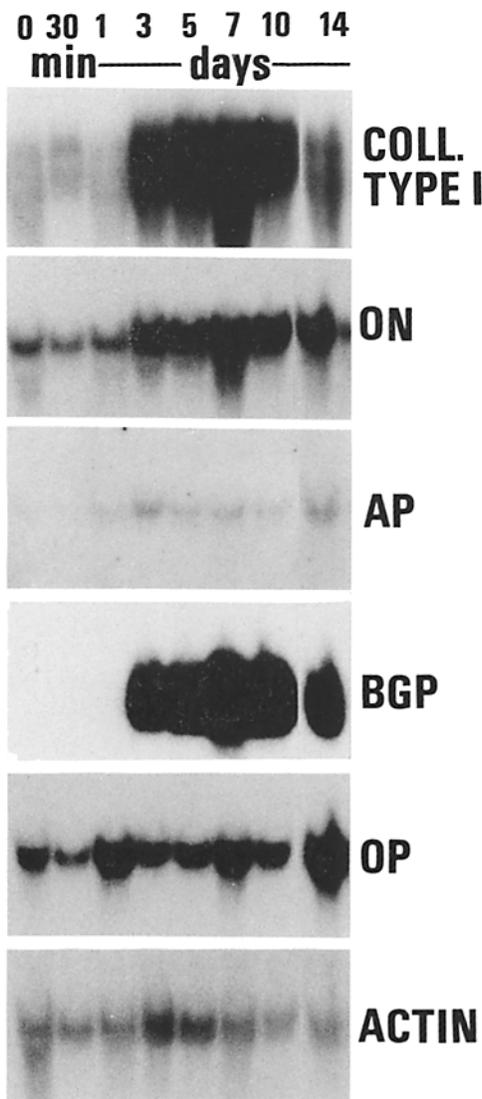


Figure 2. Enhanced expression of collagen type I (*coll. type I*), osteonectin (*ON*), alkaline phosphatase (*AP*), bone gla protein (*BGP*), and osteopontin (*OP*) in condyles during culture. Total RNA was extracted from fresh explants (*O*), and condyles cultured for 30 min or 1–14 d, and analyzed by Northern blotting. The hybridization with the indicated probes was carried out sequentially on the same filter. The filters were dehybridized completely after each hybridization. Hybridization to actin served as internal control of the amount of RNA bound to the filter. The sizes of the transcripts were: collagen type I, 4.8–5.8 kb; osteonectin, 2.2 kb; alkaline phosphatase, 2.5 kb; BGP, 0.6 kb; osteopontin, 1.5 kb; actin, 2.2 kb.

of culture, and sixfold after 10 d. Similarly, the expression of osteonectin was two- to threefold higher by day 3, and sevenfold higher after 1 wk and later. The expression of alkaline phosphatase peaked between 3 and 5 d, and the overall activation was less prominent (two- to threefold).

Expression of BGP mRNA was not detectable in RNA from total condyles before 1–2 d in culture. In general, a constant level of BGP RNA was maintained during days 3–5. In some experiments, the expression increased markedly after ~7 d (Fig. 3).

The expression of osteopontin mRNA was more complex. In independent experiments, the RNA level peaked three

times: after 1 d, 1 wk, and 2 wk of culture. After 2 wk of culture, enhancement up to eightfold could be observed.

III. Decreased Expression of a Cartilage-specific Gene (Northern Analysis)

We investigated the expression of collagen type II, which is characteristic for cartilage cells, as a parameter for changes in the expression of the chondrogenic phenotype. As shown in Fig. 3, collagen type II RNA was detected at the start, and decreased continuously to the end of the culture. In the same culture the expression of BGP RNA was induced de novo and increased with time. BGP RNA level was highest at day 7, when collagen type II RNA was no longer detectable. The osteogenic and chondrogenic gene expression patterns thus seem to be mutually exclusive.

IV. Expression of Bone Cell Characteristic Genes in Differentiating Osteoblasts and Hypertrophic Chondrocytes (In Situ Hybridization)

We applied the in situ hybridization technique to locate the areas of gene expression within the condyles and determine the type of cells in which the various genes were activated.

In condyles from neonates collagen type I RNA (Fig. 4) was expressed exclusively in the apical and lateral progenitor cells. The region of hypertrophic chondrocytes was negative for collagen type I expression. In contrast, after 2 h in culture, de novo expression of type I collagen was detected in hypertrophic chondrocytes in the basal part of the tissue. The number of hypertrophic chondrocytes positive for type I expression increased during culture. After 2 d, type I expression was detected in all areas of the condyles. Increasing type I expression was also observed in the progenitor zone, and was highest in the areas of new bone formation.

The localization of the expression of osteonectin RNA revealed a somewhat different pattern (Fig. 5). Before culture, hybridization signals were only found in the progenitor cells. After 2 h of culture, a broader area of apical progenitor cells reacted with the osteonectin probe, and after 1 d the area of positive cells extended into the zone of chondroblasts. Some

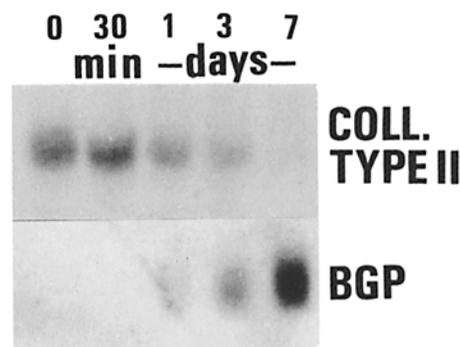


Figure 3. Decreased expression of collagen type II (*coll. type II*) during culture. Total RNA from condyles was analyzed as described in Fig. 2. The increasing expression of BGP confirms the osteogenic differentiation in these cultures. The BGP signal intensity is not comparable with that shown in Fig. 2, as the exposure times are different. The sizes of the transcripts were: collagen type II, 5.0 kb; BGP, 0.6 kb.

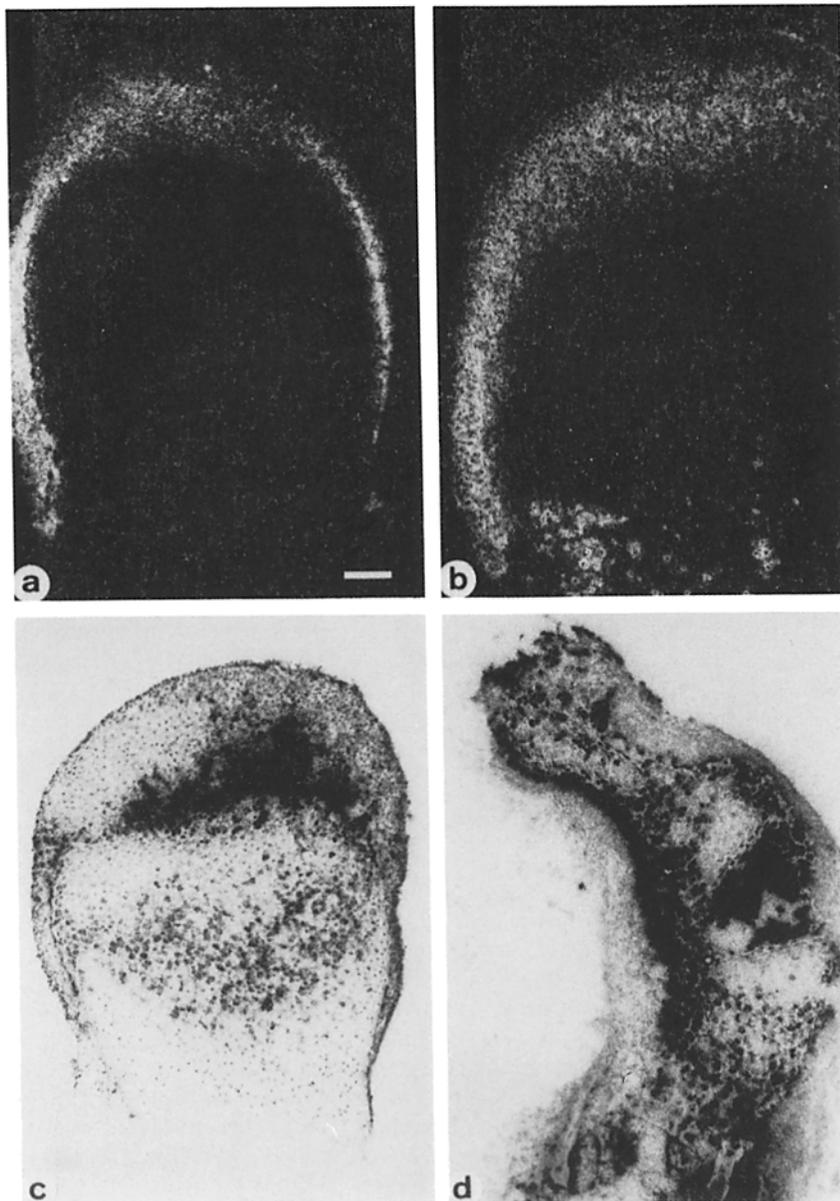


Figure 4. Collagen type I RNA distribution in condyles during culture. Autoradiographs of sections processed for in situ hybridization with the collagen type I probe, photographed with dark- (*a* and *b*) or bright-field (*c* and *d*) illumination. Fresh explant (*a*), condyles cultured for 2 hours (*b*), 2 d (*c*), and 6 d (*d*). Bar, 100 μ m.

hypertrophic chondrocytes were seen to be expressing osteonectin within 10 h of the start of the culture, and increasing numbers of positive cells were found in the first 3 d of the culture. Cells in the areas of new bone formation showed the strongest hybridization signals.

During the first 2 h of culture, alkaline phosphatase expression (Fig. 6) was only detected in the lateral progenitor cells. After 1 d, apical progenitor cells and chondroblasts, but not hypertrophic chondrocytes, also showed positive signals. After 2 d, hypertrophic chondrocytes also showed alkaline phosphatase-specific hybridization signals. The strongest hybridization was found in condyle cultures between 2 and 5 d.

BGP RNA (Fig. 7) was first found in condyles after 2 d in culture, in areas which subsequently showed osteoid formation. After 3 d, an increasing number of positive cells were observed in the zone of hypertrophic chondrocytes.

Low osteopontin RNA expression (Fig. 8) was detected in the lateral, but not apical, progenitor zone of freshly obtained condyles. In contrast to the other markers of osteogenic cells osteopontin was first activated (as early as 2 h) in the region of hypertrophic chondrocytes, and only later in the cells of the apical progenitor zone (after 3 d in culture). The whole area of hypertrophic chondroblasts and chondrocytes was positive for osteopontin RNA within 1 d of culture. After 1 wk, osteopontin RNA was detected in the areas of osteoid formation as well as in the zone of hypertrophic chondrogenic cells.

Discussion

The mandibular condyle of the newborn mouse is composed of two main cell populations: (*a*) progenitor cells, which are located in the apical and lateral zones; in vivo these cells

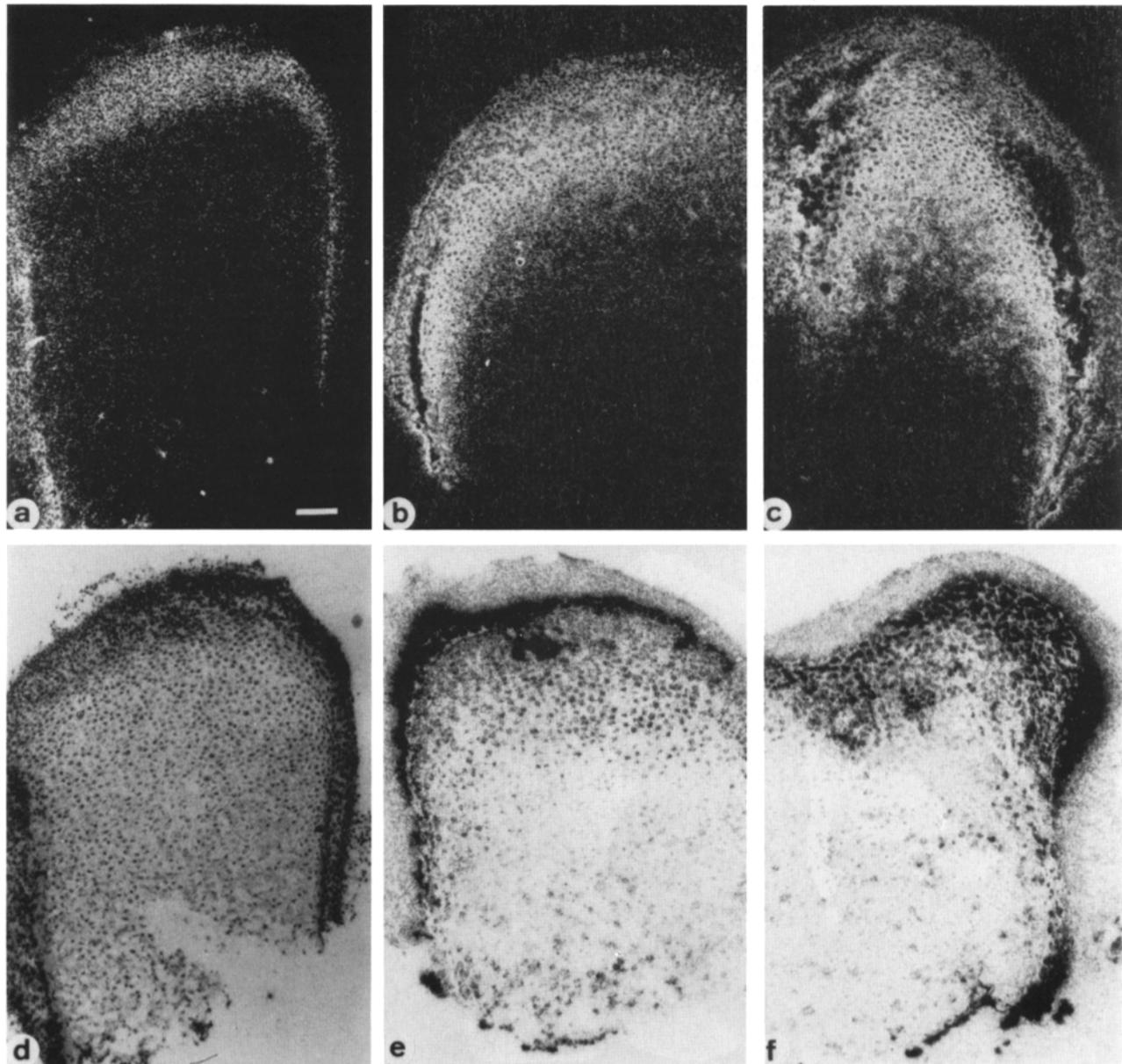


Figure 5. Osteonectin RNA distribution in condyles during culture. Autoradiographs of sections processed for in situ hybridization with the osteonectin probe, photographed with dark- (*a-c*), or bright-field (*d-f*) illumination. Slides showing less hybridization signals were photographed with dark-field illumination and slides containing strong signals were photographed with bright-field illumination. For control purposes, *a* and *d* illustrate the dark- and bright-field of the same section. Fresh explant (*a* and *d*), condyles cultured for 2 h (*b*), 24 h (*c*), 3 d (*e*), and 5 d (*f*). Bar, 100 μ m.

differentiate and become cartilage cells, under in vitro conditions cells in the progenitor zone undergo osteogenic differentiation and form new bone (Silbermann et al., 1986); (*b*) differentiated cartilage cells, which make up the interior part of the tissue (zone of hypertrophic chondrocytes, see Fig. 1).

Although the overall architecture of the tissue was maintained during culture, the cells underwent distinct changes in gene expression. Both cells in the apical progenitor zone and cells in the zone of hypertrophic chondrocytes showed *de novo* or markedly increased steady-state expression of collagen type I, osteonectin, alkaline phosphatase, osteopontin, and BGP mRNA, i.e., a pattern of gene expression typical

for osteoblastic cells. BGP is only synthesized in osteoblasts (Celeste et al., 1986; Yoon et al., 1988; Lian et al., 1989) and is thus considered as a specific marker for differentiated osteogenic cells. These results are consistent with the morphological data showing osteoid formation in this area of the condyle (Schmidt et al., 1986*b*; Silbermann et al., 1986).

In situ analysis of RNA expression in the progenitor cells in the apical area of the condyles showed a distinct temporal pattern: collagen type I and osteonectin were already expressed at the start of the culture, alkaline phosphatase was activated within 24 h, and BGP after 2 d. The expression of genes typifying osteogenic differentiation in the zone of

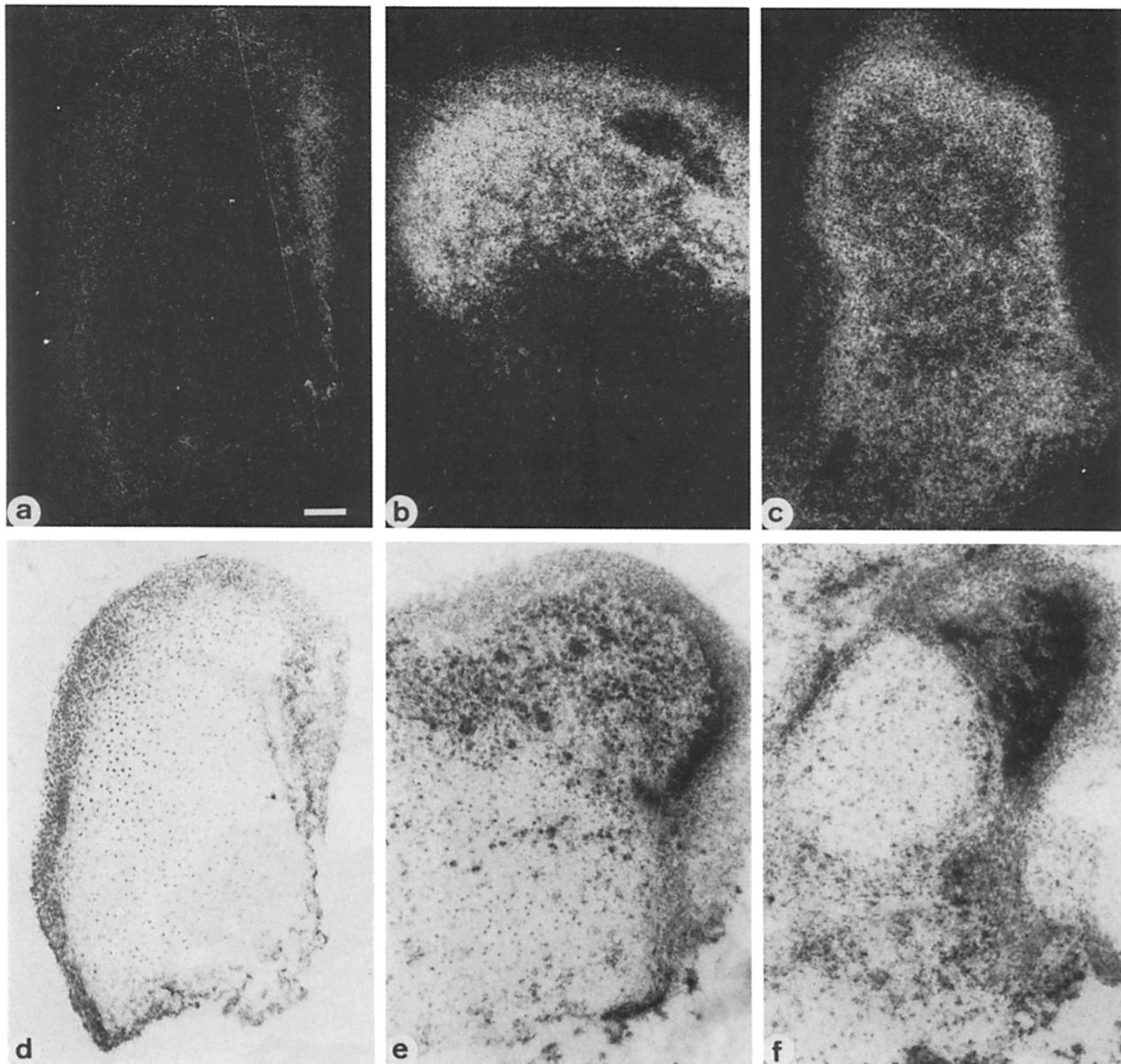


Figure 6. Alkaline phosphatase RNA distribution in condyles during culture. Autoradiographs of sections processed for in situ hybridization with the alkaline phosphatase probe, photographed with dark-field (*a-c*), or bright-field (*d-f*) illumination. Fresh explant (*a* and *d*), condyles cultured for 24 h (*b*), 3 d (*c*), 5 d (*e*), and 7 d (*f*). Bar, 100 μ m.

hypertrophic chondrocytes was delayed by ~ 1 d in comparison with progenitor cells: collagen type I RNA (after 2 h) was followed by osteonectin (10 h), alkaline phosphatase (2 d) and BGP (3 d). The temporal pattern of gene expression in both areas suggests a sequence of steady-state RNA expression during osteogenic differentiation (Fig. 9). Each gene seemed to be individually regulated, although we cannot rule out that activation of the late genes may depend on activation of the earlier ones. Similar conclusions were drawn from studies on gene expression during the development of calvaria (Yoon et al., 1987). The authors suggested a coordinated regulation of gene expression within two groups of genes. The group of genes, expressed initially, included col-

lagen type I, osteonectin, and alkaline phosphatase. The second group, which was expressed at a later stage, was formed by osteopontin and BGP.

Our data indicate that the osteopontin gene was regulated differently to the other osteoblastic markers. In the apical progenitor cells, which exhibited a more advanced stage of osteogenic differentiation, osteopontin was only detected at day 3 of the culture, i.e., at a time point when all other marker genes were already expressed at a high level. In contrast, in hypertrophic chondrocytes osteopontin expression was detected within 2 h of culture, i.e., before expression of alkaline phosphatase or bone gla protein. The highest level of osteopontin RNA was seen when the condyles were cul-

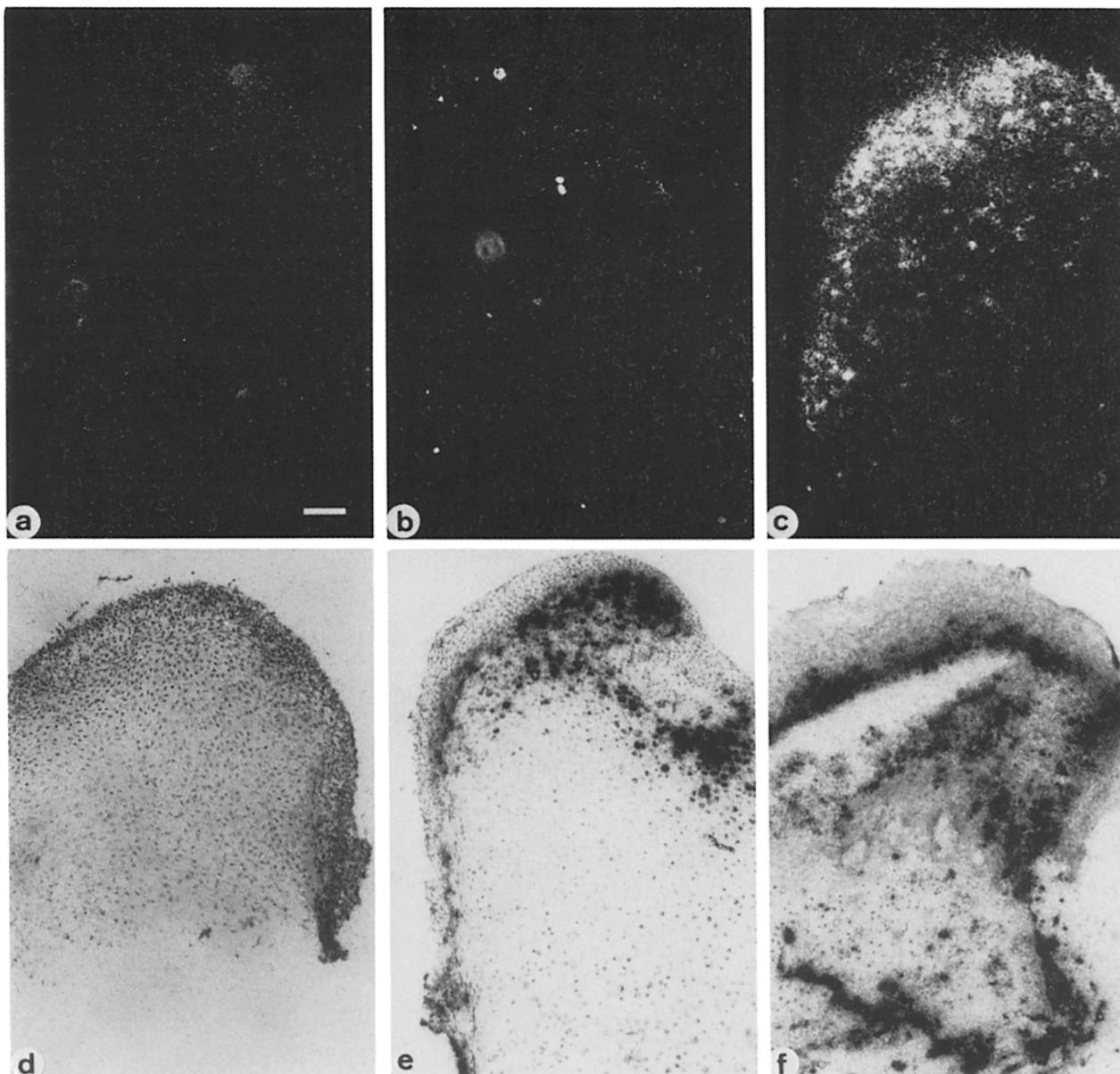


Figure 7. BGP RNA distribution in condyles during culture. Autoradiographs of sections processed for in situ hybridization with the bone gla protein probe, photographed with dark-field (*a-c*) or bright-field (*d-f*) illumination. Fresh explant (*a* and *d*), condyles cultured for 24 h (*b*), 2 d (*c*), 3 d (*e*), and 5 d (*f*). Bar, 100 μ m.

tured for 14 d. These data might explain the apparent inconsistency of previous reports describing osteopontin expression in early precursor cells (Mark et al., 1987; Nomura et al., 1988), as well as at late stages of bone development (Yoon et al., 1987).

The de novo and sequential expression of RNA may indicate that the main regulatory events during differentiation occur at the transcriptional level. Similar conclusions were drawn from the observation of sequential appearance of cartilage matrix proteins during chondrocyte differentiation (Franzen et al., 1987).

The expression of genes characteristic of osteogenic differentiation in cells in the zone of hypertrophic chondrocytes is a novel finding. There are three possible explanations: (*a*) osteoprogenitor cells invade from the periphery; (*b*) the region per se contains osteoprogenitor cells; and (*c*) mature chon-

drocytes start to express genes characteristic of osteogenic differentiation. The earliest de novo expression of genes was observed 2 (collagen type I RNA) and 10 h (osteonectin) after start of culture. At this time, there is no sign of invasion of cells into the collagen sponge and it seems unlikely that progenitor cells had already invaded the region of hypertrophic chondrocytes. Equally, in freshly obtained specimens there is no morphological indication (by light or electron microscopy) that the region contains any cells other than mature chondrocytes. Thus, the most likely explanation is that mature chondrocytes start to express genes characteristic for osteogenic differentiation. This suggestion is further supported by the concomitant observation of decreasing levels of collagen type II RNA, a specific marker for differentiated cartilage cells. Thus, it seems possible that mature chondrocytes in condyles maintain or acquire an osteogenic potential. In

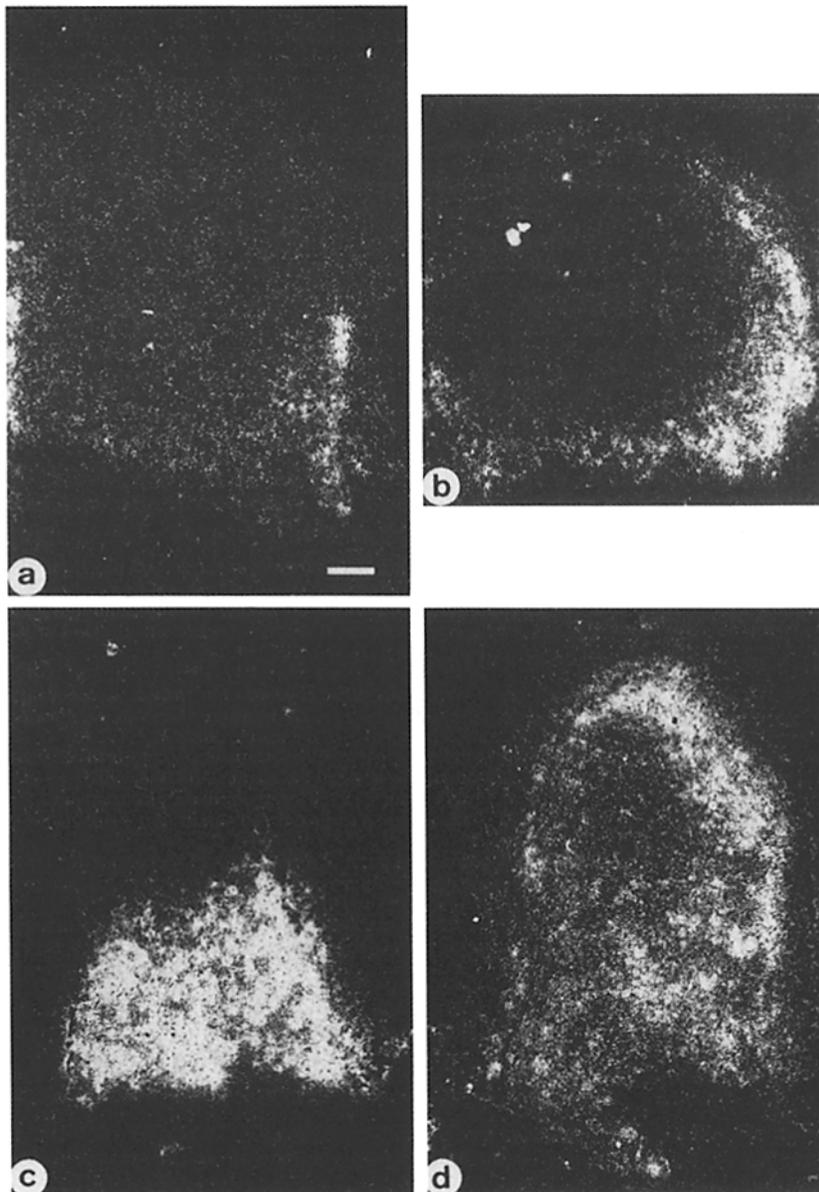


Figure 8. Osteopontin RNA distribution in condyles during culture. Autoradiographs of sections processed for in situ hybridization with the osteopontin probe, photographed with dark-field illumination. Fresh explant (a), condyles cultured for 6 h (b), 24 h (c), and 3 d (d). Bar, 100 μ m.

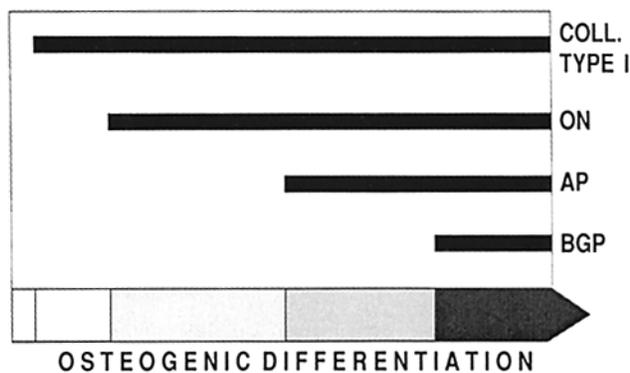


Figure 9. Schematic diagram of increasing stages of osteogenic differentiation as defined by the observed sequential activation of osteogenic marker genes.

summary, the results show that there is a specific sequential expression of characteristic genes during osteogenic differentiation in vitro. In addition, they suggest the possibility that mature chondrocytes retain or acquire the potential for osteogenic differentiation.

We thank Dr. A. B. Murray for carefully reading the manuscript and gratefully acknowledge the excellent technical assistance of R. Baier, F. Heineemann, L. Rieke, H. Schulze, and B. Zimmermann.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 324), by the Wilhelm Sander-Stiftung (Nr. 88.012.1), and by the European Atomic Energy Commission (B-I-6-0080-D (B)).

Received for publication 26 September 1989 and in revised form 19 December 1989.

References

- Brahic, M., and A. T. Haase. 1978. Detection of viral sequences of low reiteration frequency by in situ hybridization. *Proc. Natl. Acad. Sci. USA.* 75: 6125-6129.
- Caplan, A. I., G. Syftestad, and P. Osdoby. 1983. The development of embryonic bone and cartilage in tissue culture. *Clin. Orthop.* 174:243-263.
- Castagnola, P., B. Dozin, G. Moro, and R. Cancedda. 1988. Changes in the expression of collagen genes show two stages in chondrocytes differentiation in vitro. *J. Cell Biol.* 106:461-467.
- Celeste, A. J., V. Rosen, J. L. Buecker, R. Kriz, E. A. Wang, and J. M. Wozney. 1986. Isolation of the human gene for bone gla protein utilizing mouse and rat cDNA clones. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1885-1890.
- Chirgwin, J. M., A. E. Przybyla, R. J. McDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294-5299.
- Chu, M. L., J. C. Myers, M. P. Bernard, J. F. Ding, and F. Ramirez. 1982. Cloning and characterization of five overlapping cDNAs specific for the human $\alpha 1$ (I) collagen chain. *Nucleic Acids Res.* 10:5925-5934.
- Cowell, H. R., E. B. Hunziker, and L. Rosenberg. 1987. The role of hypertrophic chondrocytes in endochondral ossification and in the development of secondary centers of ossification. *J. Bone Joint Surg.* 69-A:159-161.
- Crelin, E. S., and W. E. Koch. 1967. An autoradiographic study of chondrocyte transformation into chondroclasts and osteocytes during bone formation in vitro. *Anat. Rec.* 158:473-484.
- Deschepper, C. F., S. H. Mellon, F. Cumin, J. D. Baxter, and W. F. Ganong. 1986. Analysis by immunocytochemistry and in situ hybridization of renin and its mRNA in kidney, testis, adrenal, and pituitary of the rat. *Proc. Natl. Acad. Sci. USA.* 83:7552-7556.
- Franzen, A., D. Heinegard, and M. Solorsh. 1987. Evidence for sequential appearance of cartilage matrix proteins in developing mouse limbs and in cultures of mouse mesenchymal cells. *Differentiation.* 36:199-210.
- Hafen, E., M. Levine, R. L. Garber, and W. J. Gehring. 1983. An improved in situ hybridization method for the detection of cellular RNAs in drosophila tissue sections and its application for localizing transcripts of the homeotic antennapedia gene complex. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:617-623.
- Holland, P. W. H., S. Harper, J. H. McVey, and B. L. M. Hogan. 1987. In vivo expression of mRNA for the Ca^{++} -binding protein SPARC (osteonection) revealed by in situ hybridization. *J. Cell Biol.* 105:473-482.
- Holtrop, M. E. 1966. *Calcified Tissues.* 1965. H. Fleisch, H. Blackwood, and M. Owens, editors. Springer-Verlag, Berlin. 32-36.
- Kahn, A. J., and D. J. Simmons. 1977. Chondrocyte-to-osteocyte transformation in grafts of penchondrium-free epiphyseal cartilage. *Clin. Orthop.* 129: 299-304.
- Knese, K. H., and A. M. Knoop. 1961. Elektronenmikroskopische Beobachtungen über die Zellen in der Eröffnungszone des Epiphysenknorpels. *Zeit. Zellforsch.* 54:1-38.
- Kühn, K. 1986. The collagen family: variations in the molecular and supermolecular structure. *Rheumatology.* 10:29-69.
- Kuhlman, R. E., and M. J. McNamee. 1970. The biochemical importance of the hypertrophic cartilage cell area to endochondral bone formation. *J. Bone Joint Surg.* 52A:1025-1032.
- Lewinson, D., and M. Silbermann. 1986. Parathyroid hormone stimulates proliferation of chondroprogenitor cells in vitro. *Calcif. Tissue Int.* 38:155-162.
- Lian, J., C. Stewart, E. Puchacz, S. Mackowiak, V. Shalhoub, D. Collart, G. Zambetti, and G. Stein. 1989. Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression. *Proc. Natl. Acad. Sci. USA.* 86: 1143-1147.
- Livne, E., J. Schmidt, E. I. Closs, M. Silbermann, and V. Erfle. 1989. Effects of leukemogenic retroviruses on condylar cartilage in vitro: An ultrastructural study. *Calcif. Tissue Int.* 44:25-35.
- Lutfi, A. 1971. The fact of chondrocytes during cartilage erosion in the growing tibia in the domestic fowl (*Gallus domesticus*). *Acta Anat.* 79:27.
- Maniatis, T., E. F. Fritsch, and J. Sambrock. 1982. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 200-201.
- Mark, M. P., C. W. Prince, T. Oosawa, S. Gray, A. L. J. J. Bronckers, and W. T. Butler. 1987. Immunohistochemical demonstration of a 44-KD phosphoprotein in developing rat bones. *J. Histochem. Cytochem.* 35:707-715.
- Marks, S. C., and S. N. Popoff. 1988. Bone cell biology: the regulation of development, structure, and function in the skeleton. *Am. J. Anat.* 183:1-144.
- Mason, I. J., A. Taylor, J. G. Williams, H. Sage, and B. L. M. Hogan. 1986. Evidence from molecular cloning that SPARC, a major product of mouse embryo parietal endoderm, is related to an endothelial cell "culture shock" glycoprotein of Mr 43 000. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1465-1472.
- Moos, M., and D. Gallwitz. Structure of two human β -actin-related processed genes one of which is located next to a simple repetitive sequence. 1983. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:757-761.
- Murthy, G. P. K., R. Rajalakshmi, and C. V. Ramakrishnan. 1986. Developmental pattern of alkaline phosphatase in soluble and particulate fractions of rat skull cap and femur. *Calcif. Tissue Int.* 39:185-190.
- Nomura, S., A. J. Willis, D. R. Edwards, J. K. Heath, and B. L. M. Hogan. 1988. Developmental expression of 2ar (osteopontin) and SPARC (osteonection) RNA as revealed by in situ hybridization. *J. Cell Biol.* 106:441-450.
- Sandberg, M., and E. Vuorio. 1987. Localization of types I, II, and III collagen mRNAs in developing human skeletal tissues by in situ hybridization. *J. Cell Biol.* 104:1077-1084.
- Schmidt, J., E. Livne, V. Erfle, W. Gössner, and M. Silbermann. 1986. Morphology and in vivo growth characteristics of an atypical murine proliferative osseous lesion induced in vitro. *Cancer Res.* 46:3090-3098.
- Schmidt, J., E. I. Closs, E. Livne, V. Erfle, and M. Silbermann. 1989a. Biochemical characterization of virus-induced osteosarcoma-like osseous lesion in vitro. *Calcif. Tissue Int.* 45:232-242.
- Schmidt, J., E. I. Closs, A. Luz, P. G. Strauss, E. Livne, V. Erfle, and M. Silbermann. 1989b. Induction of osteogenic maturation and neoplastic transformation of "in-vitro" differentiating skeletoblasts by c-type retroviruses from radiation-induced osteosarcomas. In *Cell Transformation and Radiation-induced Cancer.* Chadwick, K. H., C. Seymour, and B. Barnhart, editors. Adam Hilger, Bristol, New York. 239-249.
- Shimomura, Y., F. H. Wezeman, and R. D. Ray. 1973. The growth cartilage plate of the rat rib: cellular differentiation. *Clin. Orthop.* 90:246-254.
- Silbermann, M., and J. Frommer. 1972. The nature of endochondral ossification in the mandibular condyle of the mouse. *Anat. Rec.* 172:659-668.
- Silbermann, M., and J. Frommer. 1974. Electron microscopy of the secondary cartilage in mandibular condyle in the mouse. *Acta Anat.* 90:330-346.
- Silbermann, M., and G. Maor. 1985. Receptor-mediated glucocorticoid inhibition of cell proliferation in mouse growth cartilage in vitro. *Acta Endocrinol.* 108:343-350.
- Silbermann, M., D. Lewinson, H. Gonen, M. A. Lizarbe, and K. von der Mark. 1983. In vitro transformation of chondroprogenitor cells into osteoblasts and the formation of new membrane bone. *Anat. Rec.* 206:373-383.
- Silbermann, M., H. Reddi, A. Hand, and R. Leapman. 1986. In vitro differentiation of skeletoblasts and osteoblasts and the development of new bone. In *Cell-mediated Calcification and Matrix Vesicles.* Y. Ali, editor. Elsevier/North Holland, Amsterdam. 285-290.
- Silbermann, M., J. Schmidt, E. Livne, K. von der Mark, and V. Erfle. 1987. In vitro induction of osteosarcoma-like lesion by transformation of differentiating skeletal precursor cells with FBR murine osteosarcomas virus. *Calcif. Tissue Int.* 41:208-217.
- Stenner, D. D., R. P. Tracy, B. L. Riggs, and K. G. Man. 1986. Human platelets contain and secrete osteonectin, a major protein of mineralized bone. *Proc. Natl. Acad. Sci. USA.* 83:6892-6896.
- Terao, M., and B. Mintz. 1987. Cloning and characterization of a cDNA coding for mouse placental alkaline phosphatase. *Proc. Natl. Acad. Sci. USA.* 84:7051-7055.
- von der Mark, K. 1980. Immunological studies on collagen type transition in chondrogenesis. *Curr. Top. Dev. Biol.* 14:199-225.
- Wolf, H., M. Haus, and E. Wilmes. 1984. Persistence of Epstein-Barr virus in the parotid gland. *J. Virol.* 51:795-798.
- Yoon, K., R. Buenaga, and G. A. Rodan. 1987. Tissue specificity and developmental expression of rat osteopontin. *Biochem. Biophys. Res. Commun.* 148:1129-1136.
- Yoon, K., S. J. C. Rutledge, R. F. Buenaga, and G. Rodan. 1988. Characterization of rat osteocalcin gene: stimulation of promoter activity by 1,25-dihydroxyvitamin D₃. *Biochemistry.* 27:8521-8526.
- Yoshioka, C., and T. Yagi. 1988. Electron microscopic observations on the fate of hypertrophic chondrocytes in condylar cartilage of rat mandible. *J. Craniofacial Genet. Dev. Biol.* 8:253-264.