Collagen Expression, Ultrastructural Assembly, and Mineralization in Cultures of Chicken Embryo Osteoblasts

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Abstract. A newly defined chick calvariae osteoblast culture system that undergoes a temporal sequence of differentiation of the osteoblast phenotype with subsequent mineralization (Gerstenfeld, L. C., S. Chipman, J. Glowacki, and J. B. Lian. 1987. *Dev. Biol.* 122:49–60) has been examined for the regulation of collagen synthesis, ultrastructural organization of collagen fibrils, and extracellular matrix mineralization. Collagen gene expression, protein synthesis, processing, and accumulation were studied in this system over a 30-d period. Steady state mRNA levels for pro $\alpha1(I)$ and pro $\alpha2$ collagen and total collagen synthesis increased 1.2- and 1.8-fold, respectively, between days 3 and 12. Thereafter, total collagen synthesis decreased 10-fold while mRNA levels decreased 2.5-fold. In contrast to the decreasing protein synthesis after day 12, total accumulated collagen in the cell layers increased sixfold from day 12 to 30. Examination of the kinetics of procollagen processing demonstrated that there was a sixfold increase in the rate of procollagen conversion to α chains from days 3 to 30 and the newly synthe-

sized collagen was more efficiently incorporated into the extracellular matrix at later culture times.

The macrostructural assembly of collagen and its relationship to culture mineralization were also examined. High voltage electron microscopy demonstrated that culture cell layers were three to four cells thick. Each cell layer was associated with a layer of well developed collagen fibrils orthogonally arranged with respect to adjacent layers. Fibrils had distinct 64–70-nm periodicity typical of type I collagen. Electron opaque areas found principally associated with the deepest layers of the fibrils consisted of calcium and phosphorus determined by electron probe microanalysis and were identified by electron diffraction as a very poorly crystalline hydroxyapatite mineral phase.

These data demonstrate for the first time that cultured osteoblasts are capable of assembling their collagen fibrils into a bone-specific macrostructure which mineralizes in a manner similar to that characterized in vivo. Further, this matrix maturation may influence the processing kinetics of the collagen molecule.

THE processes by which mineralization is initiated and regulated in bone and other vertebrate calcifying tissues are incompletely understood. It is known, however, that a prerequisite for mineralization is the synthesis and assembly of an extracellular matrix into which mineral may be deposited. Collagen type I has been shown to be the major extracellular matrix protein of bone. It comprises between 60–70% of its organic components and between 20–30% of its total dry mass (20). Physiologically, type I collagen provides the protein basis for the architecture of bone and the scaffold into which mineral is accumulated (5, 20, 41). The importance of collagen type I in maintaining structural integrity and proper mineralization of bone has been demonstrated for one form of inherited osteogenesis imperfecta. A specific frame shift mutation in the carboxyl propeptide of pro $\alpha2(I)$ resulted in severe limb deformity, multiple bone fractures and osteoporosis (39).

An understanding of how type I collagen accumulation is

regulated during bone formation may provide insight into the mechanisms of matrix mineralization. During embryonic chick calvaria development, between days 10 and 17, collagen synthesis increases from 12 to 65% of the total protein synthesis. This increase is directly proportional to greater quantities in procollagen mRNAs, a result indicating that procollagen synthesis is controlled at a pretranslational level. After such high levels of collagen synthesis have been reached, increasing calcification of the bone is observed (33). Other studies have examined collagen synthesis and its levels of regulation in the presence of hormonal factors specifically affecting bone growth or mineralization (8, 41, 42). Factors that promote bone resorption such as PTH and 1,25 dihydroxyvitamin D_3 appear to inhibit collagen synthesis (11, 43, 45) while growth-promoting factors such as insulin stimulate collagen production (42).

The relationships between collagen synthesis and the process of matrix mineralization may be examined in vitro since
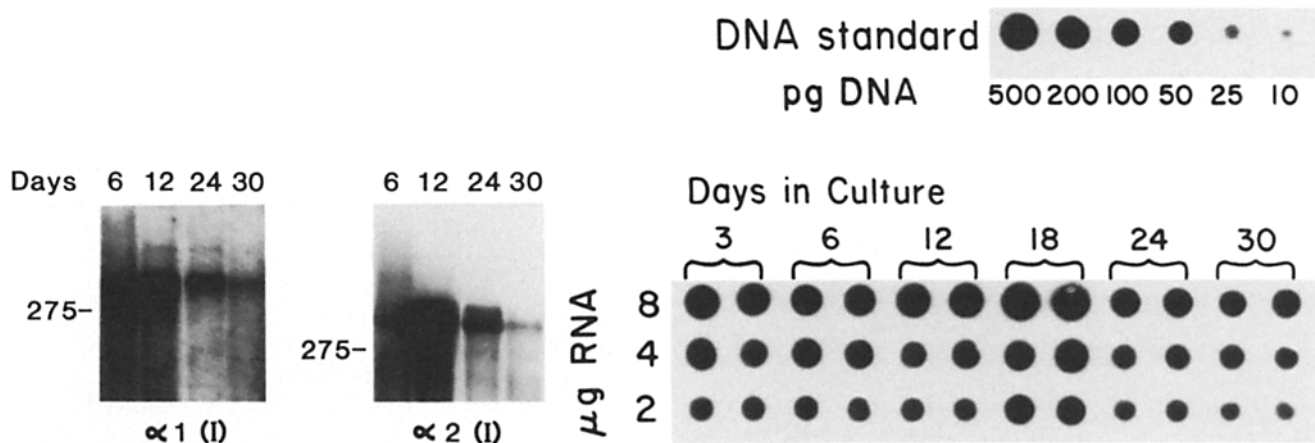
Figure 1. Hybridization analysis of steady state type I collagen mRNAs in osteoblasts. (*Left*) Northern blot analysis of either procollagen α1(I) or α2(I) mRNAs. 1 μg of poly A mRNA was loaded per lane. Culture age from which mRNA samples were obtained is denoted in the figure. Film exposure was for 24 h with an intensifying screen. (*Right*) Representative dot blot analysis of procollagen α1(I) mRNAs found in total RNA. RNA quantity applied to the filter; culture day and DNA standard are shown. Film exposure was for 24 h with an intensifying screen.

several reports have demonstrated the ability of cultured osteoblasts to produce a calcified matrix (3, 4, 10, 17, 22, 34–36, 48, 49, 55). Recently, we have shown osteoblast differentiation in vitro by a 50–100-fold increase of alkaline phosphatase enzyme activity and osteocalcin synthesis over a 30-d growth period in culture (17). During this time these cultures accumulated 60–75% of total cell layer protein as type I collagen. When grown in the presence of β-glycerophosphate (a chemical stimulant of calcification), a 20-fold increase in calcium content was measured and numerous calcific nodules were observed throughout the cultures (19). To assess the levels at which collagen protein accumulation may be regulated, we have now examined collagen gene expression, protein synthesis, and procollagen processing in this chicken embryo osteoblast culture system over the same 30-d period. In addition, the three-dimensional structural assembly of cellular and extracellular components and the chemical nature of the inorganic mineral phase deposited in this system were also studied.

Materials and Methods

Cell Culture

Osteoblasts were isolated by sequential trypsin/collagenase treatment of 17-d embryonic chicken calvariae and cells capable of expressing an osteoblastic phenotype were selected as previously described (17). Cultures were initially grown for 3 wk in minimal medium, then they were subcultivated and grown for up to 32 d in BGJb (Fitton Jackson medium supplemented with 50 μg/ml ascorbate, 10 mM β-glycerophosphate).

Protein Pulse Labeling and Pulse–Chase Analysis

Cells were pulse labeled with L(2,3,4,5) [³H]proline 120 mCi/mmol (Amersham Corp., Arlington Heights, IL) for either 24 h (steady-state synthesis analysis) or 30 min (pulse-chase analysis) as previously described (16). For pulse-chase analysis, the pulse media was removed after 30 min and cell layers were washed with 5 ml of PBS. Cell layers were then replenished with 5 ml of fresh minimal essential media and time points were collected at 15 and 30 min, 1, 2.5, 5, and 24 h. The total cell layer and media proteins for steady state or pulse-chase labelings were separately extracted (18). Proteins were analyzed by electrophoresis on 5–10% continuous gradient SDS polyacrylamide gels (23). Fluorography was carried out by the

method of Bonner and Laskey (6) and Laskey and Mills (28) and quantitation of the procollagen and collagen protein bands was carried out by scanning densitometry. The gels were dried and exposed at −50°C for varying lengths of times as described in figure legends below. Proline pulse-labeled protein (50,000 cpm) was digested with collagenase, and the amount of collagenase-sensitive material was determined by the method of Peterkofsky and Diegelmann (38). Total protein synthesis was measured by determination of the total counts per minute of acid insoluble protein.

RNA Extraction

Total RNA was extracted from 10 culture dishes (100 mm diam) at various time points by using a modification of the phenol-proteinase K method (18). Total nucleic acid samples were resuspended in 6 M guanidine HCl at 400 μg/ml, followed by the addition of 0.5 vol of absolute ethanol, and precipitated at −20°C for not more than 12 h. Total RNA was recovered by centrifugation at 8,500 g, washed in 70% ethanol, dried, and reprecipitated. This method of RNA preparation has been previously shown to separate RNA and DNA quantitatively (18). Polyadenylated mRNA was prepared as previously described (12).

RNA was subjected to electrophoresis on 25 cm agarose gels containing 2.2 M formaldehyde and blotting was carried out by the method of Thomas (50). Dot blots and mRNA copy number analysis were carried out as described previously (18). Nick translation of probes and hybridization conditions were earlier reported (12) using cDNA clones pCg45 (31), pCg54 (30), and pCg322 (56) for determination of pro α2(I), pro α1(I), and pro α1(III) collagen mRNA levels, respectively.

Ultrastructural Analysis

For ultrastructural analysis, cells in culture wells were fixed directly by either aqueous (glutaraldehyde-OsO₄) or anhydrous (ethylene glycol) methods (27). Thin tissue sections (80–120 nm) were cut using diamond knives with an Ultrotome III (LKB Instruments, Inc., Gaithersburg, MD) on a veronal acetate trough (aqueous technique) or on ethylene glycol (anhydrous method). Some sections were stained with 80% uranyl acetate in absolute ethanol and with Sato's lead. Conventional transmission microscopy was carried out at 60 KeV in a Philips 300 electron microscope, selected area electron diffraction (unstained sections) was conducted at 80 KeV in the same instrument, and electron probe microanalysis (unstained sections) was performed at 25 kV in a STEM-modified JEOL 50A scanning electron microscope (25, 26). For high voltage electron microscopy, thick sections (1–1.5 μm) prepared by aqueous or anhydrous means (27) were made using an LKB Ultrotome III and diamond knives. All sections were cut transversely through the cultures so that they contained in cross section the earliest and subsequently more recently formed layers of cells. In some instances, tissue blocks were sectioned in two directions perpendicular to each other to provide orthogonal profiles of the culture components. Some tissue sections
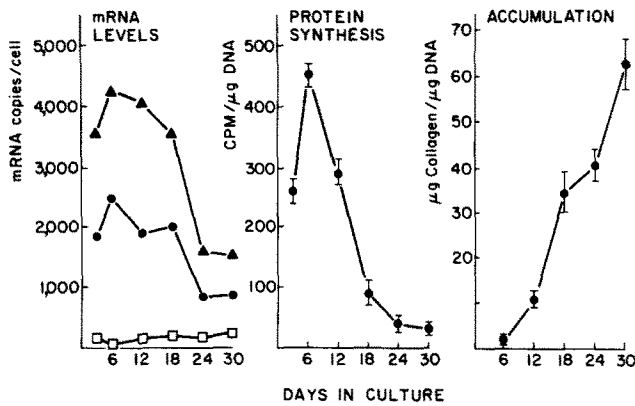
mRNA
LEVELS

mRNA copies/cell

5,000
4,000
3,000
2,000
1,000

6 12 18 24 30

PROTEIN
SYNTHESIS

CPM/μg DNA

500
400
300
200
100

6 12 18 24 30

ACCUMULATION

μg Collagen/μg DNA

70
60
50
40
30
20

6 12 18 24 30

DAYS IN CULTURE

Figure 2. Comparative analysis of total steady state mRNA levels, collagen synthesis, and collagen accumulation versus days in culture. mRNA levels, expressed as copies per cell, were derived from dot blot hybridization as previously described (18). Duplicate or triplicate culture time point mRNA samples were used for each determination. (▲) α1(I); (●) α2(I); (□) α1(III). Collagen protein synthesis was determined by bacterial collagenase digestion, as total acid precipitable cpm − acid precipitable cpm after collagenase digestion. Duplicates of four different pulse-labeled samples from each time point were used for each determination. Collagen accumulation was determined from amino acid analysis and calculated on the basis of total hydroxyproline content and ratio of hydroxyproline to proline.

were stained anhydrously with OsO_4 vapor for 30 min (29). Sections were examined at 1.0 MeV in the high voltage electron microscope located at the National Institutes of Health Biotechnology Resource Laboratories in Albany, NY. The microscope is equipped with a side-entry goniometer specimen holder maintained at −120°C (21).

Results

Collagen Gene Expression and Protein Synthesis

To determine the level at which collagen accumulation is regulated during osteoblast growth in culture, collagen gene expression and protein synthesis were examined. Collagen mRNA levels were qualitatively examined by Northern blot analysis to determine the integrity of the isolated mRNAs, their size, their relative quantity, and the distribution of the mRNAs between their various polyadenylation species. As can be seen in Fig. 1, *left* very high concentrations of procollagen α1(I) and α2(I) mRNAs were detected on days 6 and

12, after which a large decline in their amounts was observed. Scanning densitometry showed that there was considerable variation in the ratio of the various polyadenylation species at later time points in these samples with an apparent loss of the larger molecular weight mRNAs.

Absolute RNA amounts per cell can be obtained by dot analysis using total RNA (18). Since the quantities of total RNA and total DNA contents (17), the probe lengths, and probe specific activities are known for each dot blot assay, the hybridizable counts per minute are directly proportional to the absolute number of complementary sequences in the RNA sample. The results of a representative analysis for procollagen α1(I) mRNA is shown in Fig. 1, *right*. As can be seen, duplicate samples at three concentrations were measured for days 3, 6, 12, 18, 24, and 30. These results are comparable with those obtained by Northern blot analysis. At later time points, however, as large a decline in mRNA levels was not seen for the total RNA samples as was seen for poly A selected mRNAs.

A comparison of the mRNA levels to protein synthetic levels and collagen accumulation is shown in Fig. 2. Fig. 2, *left* represents a compilation of dot blot data for procollagen α1(I), α2(I), and α1(III) mRNAs, while Fig. 2, *center* panel depicts protein synthetic levels as determined by collagenase sensitivity. Analysis of mRNA levels here detected from 0.3 to 3.0% of the total collagen mRNAs as type III collagen transcripts. A summary of these data is presented in Table I in which the α1/α2 ratio of type I, the type I to type III collagen ratio, and the relative translational efficiency of the type I collagen mRNAs are compared. As can be seen from Fig. 2 and Table I, the α1/α2 ratio varies from 1.7 to 2.1 with an overall average of 1.9. This is consistent with the observed α1/α2 collagen protein ratio of 2.0 in these cells (17). Fig. 2, *right* depicts collagen accumulation determined by protein hydrolysis and total hydroxyproline quantitation. Although collagen mRNA and protein synthesis are declining after day 12, total collagen accumulation shows its most rapid increase (greater than sixfold) after day 12.

Collagen Secretion and Procollagen Processing

A comparison of the data from Fig. 2 demonstrated that collagen accumulation in culture was not controlled primarily at a pretranslational or protein synthesis level but at some posttranslational level. One possible level at which collagen accumulation could be controlled was at the level of procollagen processing. Initial experiments were therefore directed at determining if the distribution of procollagen, its process-

Table I. Collagen Synthesis and mRNA Translational Efficiency

Days in culture	α1(I)/α2 mRNA ratio*	Type III/type I mRNA ratio*	Translational efficiency‡	Percent collagen synthesis§
3	2.00	0.03	6.8	5.4 ± 1.5
6	1.70	0.01	6.4	6.5 ± 1.0
12	2.20	0.004	4.4	5.6 ± 1.0
18	1.75	0.018	1.5	8.5 ± 1.2
24	2.15	0.014	1.5	7.3 ± 0.6
30	1.75	0.015	1.1	10.6 ± 0.6

* Derived from Fig. 2 on RNA levels.
‡ Derived from Fig. 2 CPM collagenase-sensitive protein/copies of mRNA × 100.
§ Derived from collagenase-sensitive protein determination and total incorporation using method of Peterkofsky and Dieglemann (38) to calculate percent collagen synthesis.
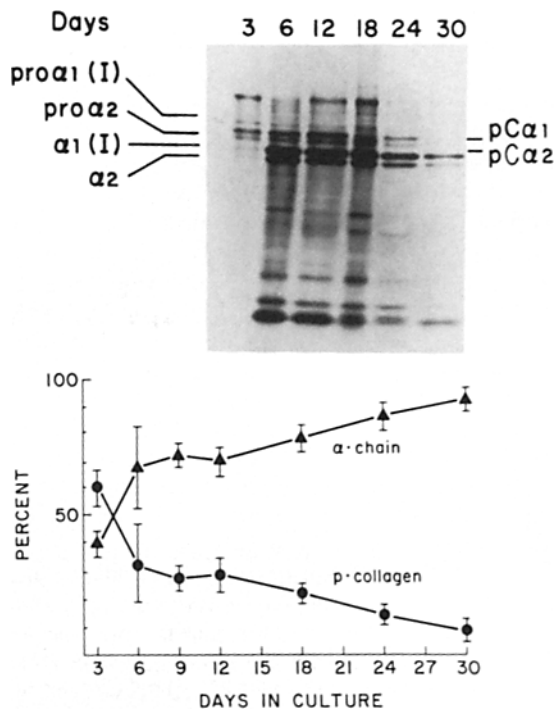
Figure 3. Steady state procollagen synthesis and processing as a function of time in culture. Procollagen, its partially processed intermediates, and α chains secreted into the culture media were separated on a 5–10% continuous gradient SDS–polyacrylamide gel. The molecular nature of the intermediates as the collagen containing the carboxyl extension peptide (pC) or the amino extension peptide (pN) was based on their mobility in comparison to data previously published for type I collagen produced by chick calvaria organ culture (9) and was consistent with previous data for type I collagen synthesized by calf smooth muscle cells (16). The percent distribution of procollagen and partially processed intermediates to α chains versus time is seen in the graph. Each time point determination was based on the average of the densitometric scans of three different gels derived from two separate time course experiments. Fluorograph exposure was for 2 d.

ing intermediates, and α chains varied throughout the 30-d culturing period. Procollagen processing profiles and the percent distribution of the pro and partially processed intermediates to α chains are shown in Fig. 3. In this experiment, cells were labeled for 24 h and the media of the cultures were analyzed by SDS-PAGE. These results clearly show that, with culture time, a greater percent of procollagen is converted to α chains during a 24-h period. The comparison of the percent distribution from day 3 to day 30 shows that there is a sixfold increase in the steady state amount of α chains secreted into the medium.

Since this experiment was performed on 24-h pulse-labeled samples, only steady states of the procollagen, its processed intermediates, and α chains may be observed. To determine procollagen processing kinetics, a pulse-chase analysis was carried out at either day 3, 12, or 30 of the culturing period. The electrophoretic profiles of the media and the cell layers of the day 3 and 30 pulse-chase experiment are shown in Fig. 4. A comparison of both media and cell layer profiles of days 3–30 demonstrates that at day 3, even after 2.5 h, most of the collagens are observed in the procollagen or partially processed forms. In contrast, at day 30 al-

most all of the procollagen and its partially processed intermediates have been chased to α chains after 0.5 h. Fig. 4 also shows that at day 3 a large proportion of the collagen molecules moves from the cell layer compartment to the medium. At day 30, however, there appears to be an equal distribution of collagen between the cell layer and the media compartments with fully processed collagen chains appearing in both compartments.

To quantitate pulse-chase data and determine the kinetics of processing of the procollagen molecules, scanning densitometry was performed on the autoradiograms shown in Fig. 4. These results are shown in Fig. 5, *A* and *C*. The $t_{1/2}$ of conversion at day 3 is ~3 h, while only ~80% of the procollagen is fully processed after 24 h. At day 30, however, the $t_{1/2}$ is <0.5 h and nearly complete processing is seen after 24 h. Since previous data have shown that collagen molecules secreted into the media do not become incorporated into the growing matrix in the cell layer (47), it was of interest to determine if many more synthesized collagen molecules became matrix associated with time. The percent distribution of the collagen between the media and cell layer compartment was obtained from the total [³H]proline collagenase–sensitive counts associated with each compartment and were roughly comparable to the gel profiles of Fig. 4. On day 3 these data show that, as the chase time increases, a greater percentage of the collagenase-sensitive material is found in the media, and after 24 h >60% of the collagenous proteins were found in this compartment. In contrast, at day 30, the amounts of collagenous proteins found in the cell layer and media compartments remain relatively constant at ~70 and ~30%, respectively. Thus, data from Fig. 5 indicate that collagen was both more efficiently processed and incorporated into the extracellular matrix at later times in culture.

Macrostructural Assembly and Mineralization of Collagen

Biochemical data (Figs. 1–5) would suggest that, by day 30, the osteoblast cultures have accumulated and assembled an extensive collagenous matrix. However, these analyses cannot provide a determination of how the synthesized collagen is packaged into fibrils and fibers, or how these might be macrostructurally organized. These parameters were assessed by microscopic examination of extracellular matrix ultrastructure. Thin sections of 30-d cultures cut parallel to the culture dish surface demonstrated that an extensive collagen matrix composed of well-developed collagen fibrils had been formed (Fig. 6). Most of the fibrils observed in these sections appeared to be aligned with their long axes in a plane parallel to the culture dish surface. Electron opaque areas of mineral (see below) were seen randomly distributed throughout the matrix and in many areas appeared to be associated with the collagen fibrils.

To obtain a three-dimensional view of the macrostructural assembly of the collagen matrix, high voltage electron microscopy of thick sections was used. These sections were cut perpendicular to the culture dish surface so that a profile of all cell layers could be examined simultaneously. Two montages of these results are presented in Figs. 7 and 8. Fig. 7 shows that the osteoblast cultures become arranged in up to three to four cell layers over a 30-d period and individual cells are embedded in a dense meshwork of collagen fibrils. Each cell layer appears to be associated with a layer of fibrils
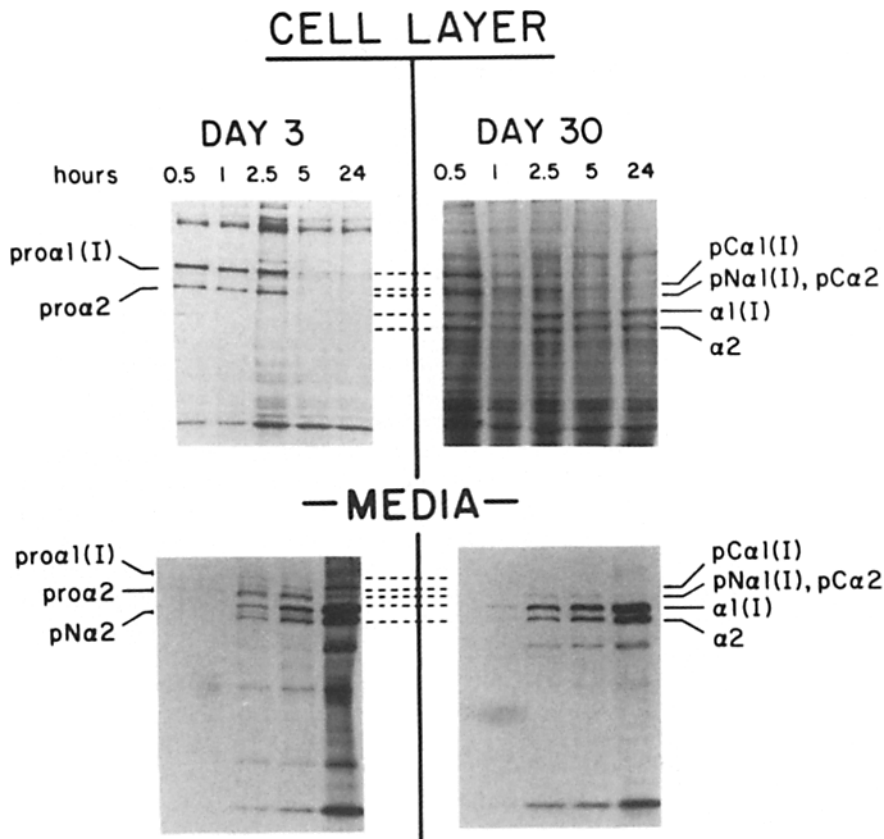
CELL LAYER

DAY 3 | DAY 30

hours 0.5 1 2.5 5 24 | 0.5 1 2.5 5 24

proα1(I)
proα2

pCα1(I)
pNα1(I), pCα2
α1(I)
α2

—MEDIA—

proα1(I)
proα2
pNα2

pCα1(I)
pNα1(I), pCα2
α1(I)
α2

Figure 4. Pulse–chase analysis of procollagen processing and secretion. Procollagen, its partially processed intermediates, and α chains were separated on a 5–10% continuous gradient SDS-polyacrylamide gel. The day of the experiment, the time of chase, and the culture compartment (media or cell layer) are denoted in the figure. Fluorograph exposure was for 6 d. Nomenclature for collagen intermediates is given in Fig. 3.
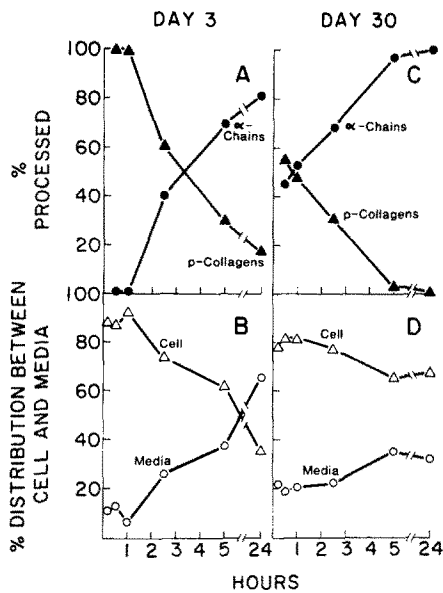
DAY 3 | DAY 30

% PROCESSED

A
α-Chains
p-Collagens

C
α-Chains
p-Collagens

% DISTRIBUTION BETWEEN CELL AND MEDIA

B
Cell
Media

D
Cell
Media

HOURS

1 2 3 4 5 24   1 2 3 4 5 24

Figure 5. Kinetic analysis of procollagen processing and secretion. The day of the experiment and time of the chase are shown. (*Top* panels) The percent distribution of procollagen and partially processed intermediates to the α chains. The distribution was determined by scanning densitometry of the gel profiles depicted in Fig. 4. An average of both the media and cell layer profiles was used for the determination. (*Bottom* panels) The percent distribution between cell layer and media of newly synthesized collagen. Total bacterial collagenase sensitive cpm were determined for combined cell layer and media. The bacterial collagenase sensitive cpm were determined separately for each compartment (media or cell layer). The percent distribution was then calculated for the collagenase sensitive cpm between the cell layer and the media.

alternately oriented at approximately right angles. In any individual layer, longitudinal axes of the fibrils are parallel. From transverse tissue sections, most fibrils were found to have diameters in the range of 40–150 nm, the fibrils adjacent to cell plasmalemma being the smaller in diameter and less well organized. The collagen fibrils of the deepest cell layer adjacent to the culture dish surface were observed to be the most extensively mineralized (Fig. 8).

Cells observed in cross-sectional profile were generally flat and elongated and contained well-defined nuclei and nucleoli. Extended cytoplasmic processes were observed between cells throughout the matrix. Cells appeared to be biologically active and viable as evidenced in part by well-developed rough endoplasmic reticulum.

To examine the spatial relationship between mineral deposits and the organic components of the extracellular matrix of the cultures, thin sections were studied by conventional electron microscopy (Fig. 9). The characteristic 64–70-nm banding pattern typical of type I collagen was observed. Relatively few extracellular membrane-bound structures such as matrix vesicles (2, 7) were found. Electron-dense areas were principally associated with collagen fibrils. Particles within the dense deposits had a needle- or platelike habit and in several areas they were deposited along collagen fibrils in a periodic manner corresponding to that of the fibrils themselves (64–70 nm). Fig. 9, *upper left inset* illustrates a typical energy dispersive x-ray spectrum generated from electron dense deposits of the cultures prepared by anhydrous means. Characteristic peaks representing calcium and phosphorus with Ca/P molar ratios in the range of 1.1–1.4 were detected. Selected area electron diffraction of dense deposits
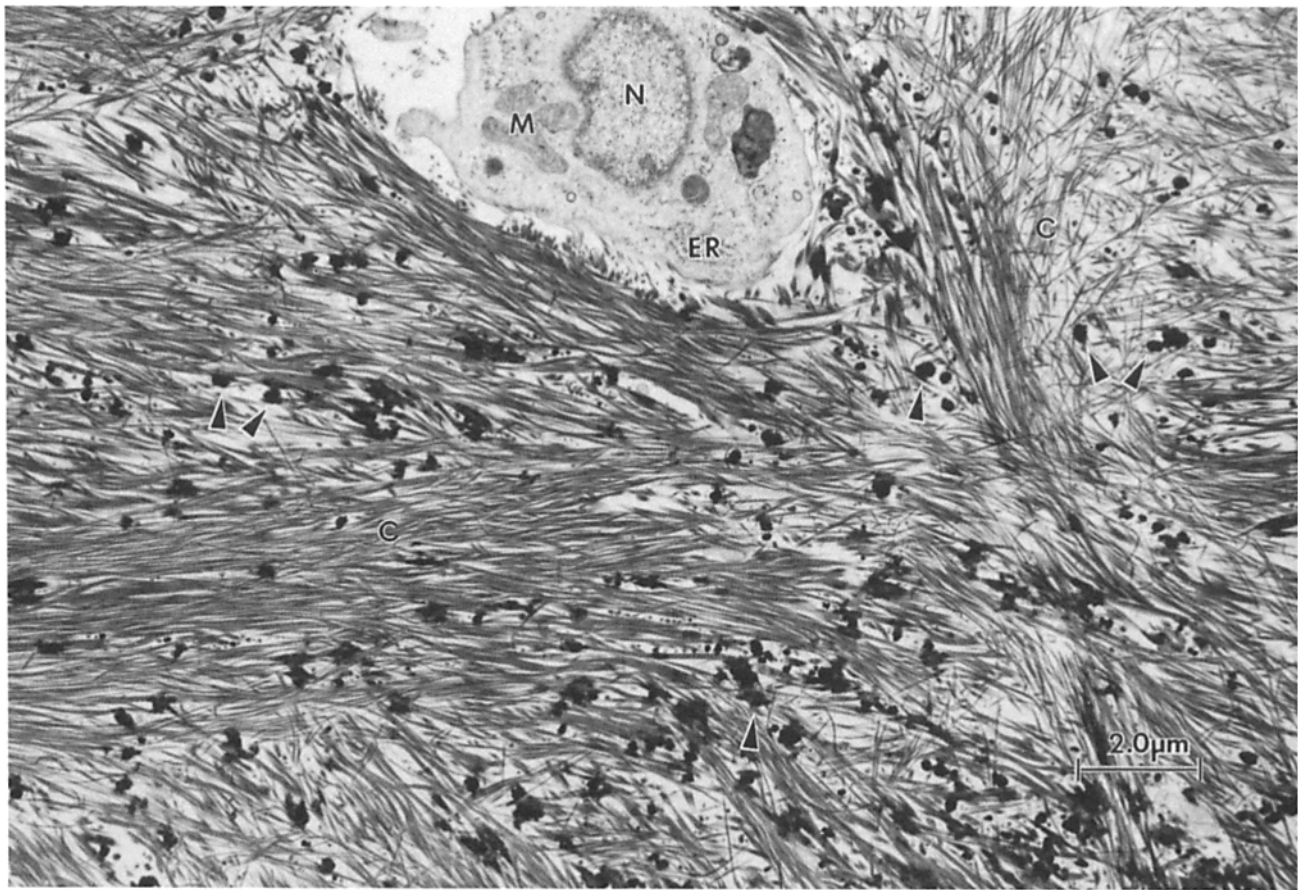
Figure 6. A relatively low magnification electron photomicrograph of a region from cultured chicken embryo osteoblasts, fixed with aqueous glutaraldehyde-osmium tetroxide, and stained with uranyl acetate and Sato's lead. The micrograph illustrates a typical cell containing a prominent nucleus (*N*), mitochondria (*M*), and endoplasmic reticulum (*ER*). The cell resides in a small lacuna surrounded by an extensive extracellular matrix characterized by long, thin fibrils of collagen (*C*) and numerous electron-dense mineral deposits (*arrowheads*) developing at this stage of culture (32 d). This section was made parallel to the surface of the culture dish in distinction to that shown in figures below.

(Fig. 9, *upper right inset*) indicated a poorly crystalline hydroxyapatite.

Discussion

In the present studies, the molecular levels of regulation for collagen synthesis and accumulation are defined in cultured chicken embryo osteoblasts. During the first 6 d of growth in culture, steady state mRNA levels and protein synthesis increased 1.2- and 1.8-fold, respectively, indicating control of protein synthesis at a pretranslational level. The elevated levels of mRNA and protein synthesis in culture are analogous to embryonic chicken calvariae bone development between days 10 and 16 in which there is also a 1.8-fold increase in both mRNA and protein synthesis (33). High collagen synthetic activity precedes matrix calcification in vivo and in vitro and would indicate that an initial collagen matrix must be formed before mineral deposition can occur.

Examination of the collagen mRNA ratios from Table I indicates at all time points that $\alpha 1(I)$ and $\alpha 2(I)$ mRNAs are coordinately regulated at a pretranslational level to maintain an approximately 2:1 ratio. While delayed reduction analysis of the newly synthesized collagen had previously failed to detect collagen type III (17), the more sensitive analysis of mRNA levels described here indicates small amounts (0.3–

3%) of the total collagen mRNAs are type III. These low levels of type III may be produced by a small number of contaminating periosteal fibroblasts or may be derived from undifferentiated mesenchymal cells. The latter explanation is consistent with the results of Reddi et al. (44) in which collagen type III was found associated with initial mesenchymal ingrowth in bone matrix-induced endochondral differentiation.

At early time points in the osteoblast culture system, there is a much greater efficiency of collagen mRNA translation than at later time points, and, while an observed 10-fold decrease in protein synthesis is seen from day 12–30, there is only a 2.5-fold decrease in the observed levels of the mRNAs. Translational control has been previously observed in vivo as well as in several other in vitro systems (15, 45, 51). This may be a result of the long half-life for these mRNAs with a subsequent loss of translation, possibly related to the loss of poly A–containing species at later time points (See Fig. 1, *left*, time points 24 and 30 d). Both procollagen $\alpha 1(I)$ and $\alpha 2(I)$ mRNAs exhibited multiple molecular weight species identical to those previously observed in numerous other tissues and cells including chondroblasts (12), myoblasts (18), and chicken calvariae (1). These multiple molecular weight species have been previously shown to be the result of multiple polyadenylation sites at their 3' ends
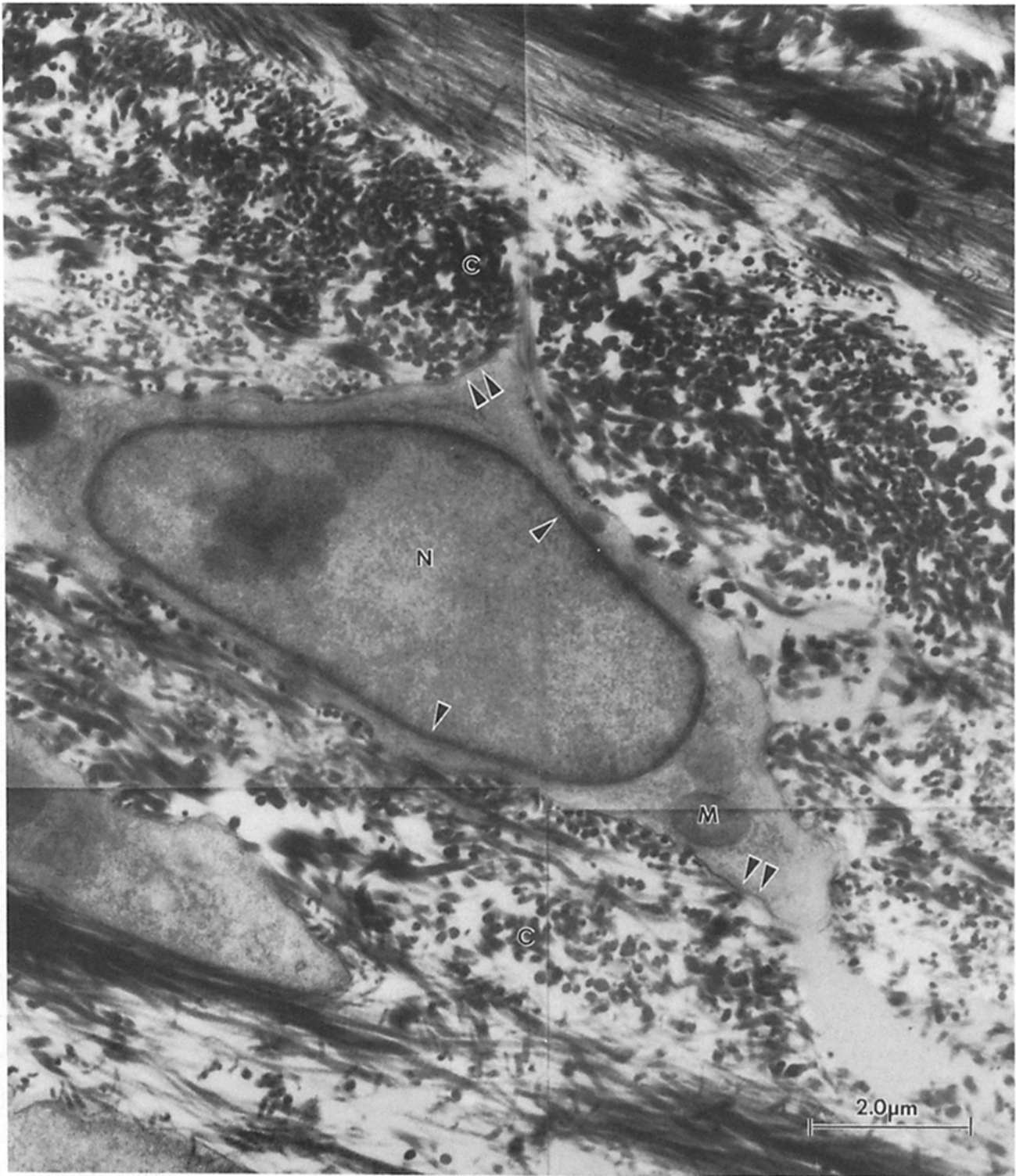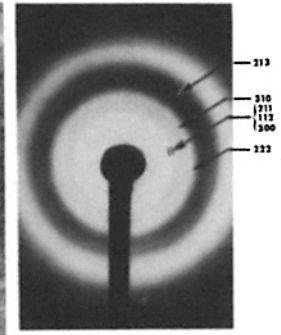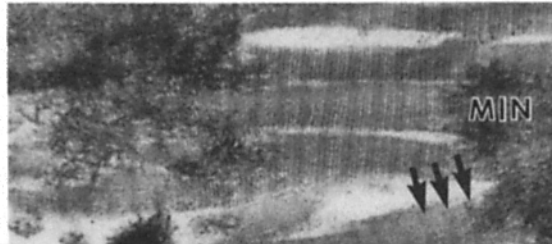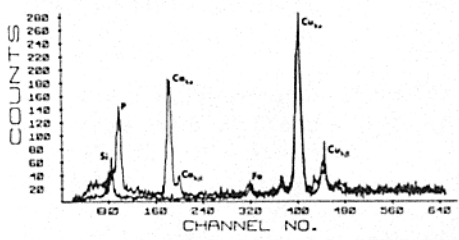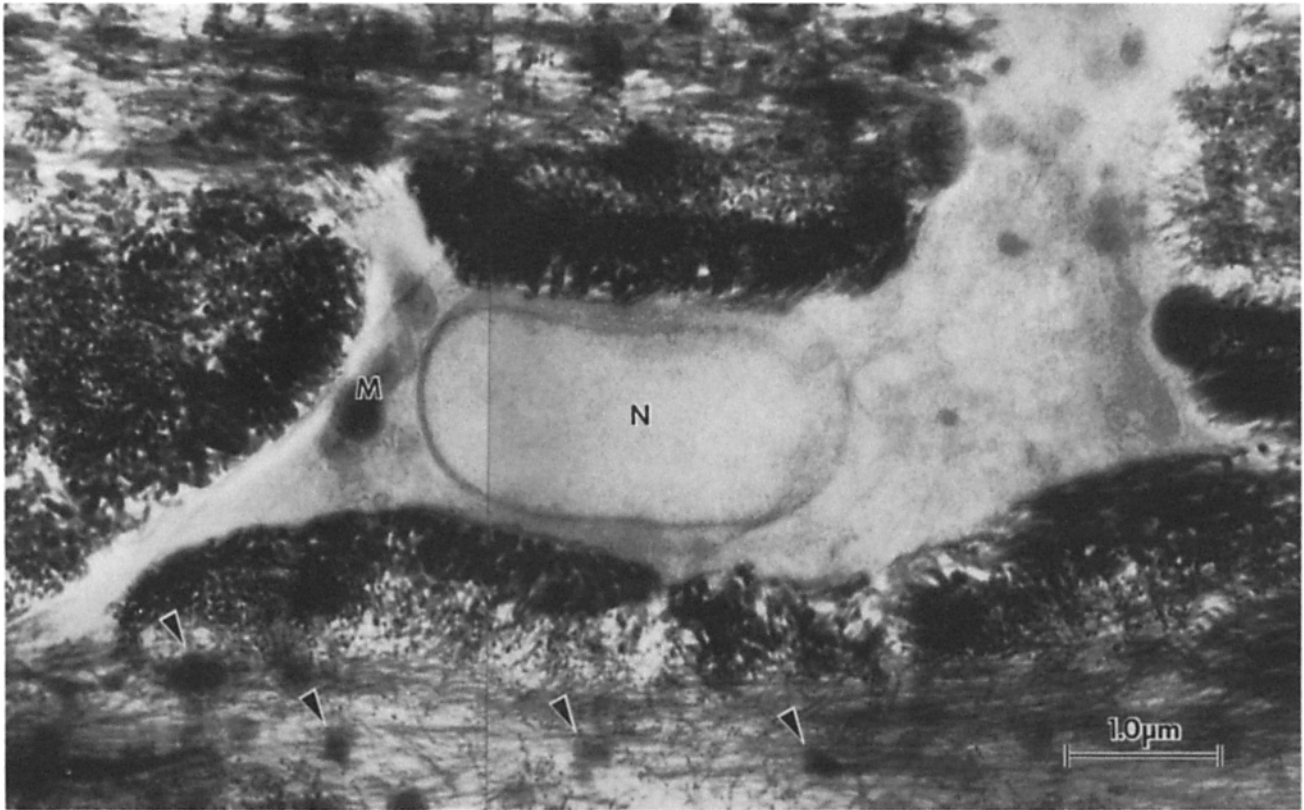
Figure 7. A montage of electron photomicrographs obtained using high voltage electron microscopy (1 MeV) of a 30-d-old osteoblast culture fixed with glutaraldehyde-osmium tetroxide and stained with uranyl acetate and Sato's lead. The 1.5-μm-thick section was cut perpendicular to the surface of the culture dish so as to provide a transverse view through the thickness of the culture. The micrograph illustrates a typical osteoblast at this stage of culture development. It contains a prominent nucleus (*N*), mitochondria (*M*), and definitive nuclear (*single arrowheads*) and cell (*double arrowheads*) membranes. An extensive extracellular matrix is characterized principally by type I collagen fibrils (*C*). It is of interest in this and other montages that culture cell layers were typically three to four cells thick and collagen fibrils were arranged orthogonally from layer to layer.

Figures 8 and 9. (Fig. 8) An electron photomicrograph obtained by high voltage electron microscopy (1 MeV) of a 30-d chick osteoblast culture fixed and stained as described in Fig. 7. The culture was sectioned perpendicular to the surface of the culture dish. Electron-dense minerals (*arrowheads*) are more numerous in association with the layer of collagen fibrils nearest the bottom of the culture dish. Thus

(1). In the present experiments, considerable variation was seen in these polyadenylation species suggesting a possible role in either mRNA stability or translational control. It is also interesting to point out the results of Paglia et al. (37) that demonstrated selective inhibition of collagen translation in vitro by the amino-terminal propeptide of type I collagen. The possible role of the amino propeptide in vivo might be indicated by the more efficient collagen processing observed in the osteoblast cultures at later time points. At these later times, higher levels of the amino propeptide would be present, and it is at these later times that low translational efficiency is observed.

A direct relationship between collagen synthesis and collagen accumulation in the cell layer was not observed. Instead, the data presented in Figs. 3–5 would indicate that collagen accumulation is largely controlled at the level of fibril assembly and procollagen processing. Fibril assembly has been shown to be a multistep process (52, 54). Several discrete phases have been identified including the formation of subfibrillar aggregates with subsequent steps involving both linear and lateral fibril growth (54). The relationship between procollagen processing and both linear and lateral fibril assembly has been suggested by several recent studies (13, 14).

Analysis of chicken embryo skin of different ages with antibody directed toward the amino extension peptide of collagen type I indicated that during early embryo development there is a much higher ratio of pNα1(I) to α1(I) (13). On localization, antibody reactivity had a specific 60-nm periodicity and was predominantly associated with thin (10–35 nm) fibrils (13, 14). These results have led to the suggestion that the amino-terminal propeptide may play a role in longitudinal fibril assembly and provide a mechanism of controlling lateral fibril growth (13, 14). Removal of the carboxyl propeptide has also been shown to be a prerequisite for fibril formation in vitro (32). The data presented here provide a direct measurement by pulse–chase analysis of processing kinetics versus collagen incorporation into an extracellular matrix and conclusively demonstrate the relationship between matrix maturation and the rate of procollagen processing.

Two possibilities exist to explain the observed differences in processing kinetics: (a) alterations in enzyme concentration; (b) alteration in the rate constant (K_m) of the enzyme. The former explanation seems less likely than the latter since it implies that the enzymes associated with procollagen processing would not be coordinately expressed with procollagen synthesis. An altered K_m, however, could result from either a more active enzyme conformation or a higher substrate concentration. Electron microscopic studies have documented that procollagen processing and early fibrillogenesis are initiated at the cell surface in the extracellular matrix (53). The data presented here show similar small fibrils adjacent to the cell plasmalemma and would tend to support these earlier findings. Thus, the processing enzymes themselves may need to be properly assembled in the matrix to have the most favorable conformation for maximal activity. Conversely, if the substrate is an early fibrillar aggregate, the rate limiting factor would be fibrillar aggregate formation, which itself may be promoted by the state of matrix maturation.

Numerous ultrastructural studies have demonstrated that collagen fibrils in situ are macrostructurally arranged in a tissue specific fashion (for example, tendon has parallel arrays while bone collagen is orthogonally arranged) (24, 54). The experiments presented here demonstrate that osteoblasts in vitro are capable of assembling their collagen fibrils in the phenotypic manner seen in situ. Whether the fibril assembly is a vectorial secretion process, as suggested by Trelstad and Hayashi (53), or is influenced by a noncollagen or minor collagen matrix molecule(s) to promote the orthogonal arrangement is unclear at this time. However, these results indicate that molecular packing of the collagen fibrils and their macrostructural assembly are intrinsic properties of this osteoblast phenotype.

On the basis of von Kossa staining for phosphate deposition or the presence of electron dense areas in electron micrographs, several laboratories have demonstrated that β-glycerophosphate promotes the calcification of the extracellular matrix produced by osteoblasts (4, 34, 35, 49, 55). These studies definitively show by electron probe microanalysis that the mineral is composed of Ca and P with relatively low Ca/P ratios. The diffuse reflections on electron diffraction indicate the mineral is a poorly crystalline hydroxyapa-

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the development of mineral and its interaction with collagen in this system are related spatially, and presumably temporally, with the progressive thickening of culture layers. The cell contains a nucleus (N) and numerous other organelles including putative mitochondria (M). Note the orthogonal nature of adjacent collagen fibril layers. (Fig. 9) A higher magnification electron photomicrograph of the extracellular matrix taken from a 30-d culture, fixed with glutaraldehyde-osmium tetroxide, and stained with uranyl acetate and Sato's lead. The extracellular matrix is replete with collagen fibrils characterized by their typical 64–70-nm periodicity. Crystallites of a mineral phase (MIN) are clearly associated with the collagen fibrils appearing in longitudinal or transverse profile (C). Along occasional longitudinal profiles, the mineral deposits, themselves, are periodic (64–70 nm) (arrows). In some places (arrowheads) the deposition occurs in extrafibrillar spaces of the matrix in a form which is spherulitic (in three dimensions) rather than in the form of discrete, oriented needle- or platelike mineral deposits associated with collagen fibrils. Such spherulites appear to be associated with collagen sectioned transversely. A portion of an osteoblast is shown (OB). (Upper left inset) Typical electron probe spectra of a single, electron-dense extracellular deposit (upper trace) and the relatively electron-transparent matrix (lower trace) adjacent (within 20 nm) to the deposit. The curves were obtained from a section of 32-d chick osteoblasts in cultures treated with ethylene glycol and left unstained so as to maintain the nature of the mineral phase (27). Electron-dense deposits analyzed were similar in appearance to those illustrated in Fig. 9 photomicrograph. Electron probe microanalysis was performed with an accelerating voltage of 25 KeV and a probe current of 1×10^{-11} A using a stationary-beam spot for 200-s counting times. The probe beam diameter was <10 nm for analysis (25). The abscissa is calibrated relative to x-ray energy (20 eV/channel). A comparison of the two spectra shows that the dense deposit consists principally of calcium and phosphorus having a Ca/P ratio on the order of 1.1. Silicon in the matrix spectrum originates from slight specimen contamination on microanalysis, copper derives from the grid supporting the section, and iron from the pole piece of the electron probe. (Upper right inset) Selected area electron diffraction pattern from a small cluster of electron-dense material in the 32-d osteoblast culture treated by anhydrous means. Electron diffraction was performed at 80 KeV and reflections were calibrated against a gold standard examined under the same conditions. The poorly resolved, indexed reflections are characteristic of a very poorly crystalline hydroxyapatite.

tite. These data are similar to those observed in situ in newly mineralizing tissue (24–26, 29, 40).

In sections in which all the cell culture layers are examined simultaneously, it is of interest to note that the deepest collagen fibril layer was most heavily mineralized. Whether this layer of collagen is the first synthesized is still unclear since it is not understood how the matrix is assembled temporally. Similar observations have been reported by Nefussi et al. (34) in cultures of mouse calvaria osteoblasts. While mineral is predominantly associated with collagen fibrils during culture development, some membrane-bound, vesicular structures containing Ca and P were also observed at early culture time points and in cultures without β-glycerophosphate supplementation (Gerstenfeld, L. C., and W. J. Landis, unpublished results). In size and shape, such vesicles resemble those observed in situ (2, 7), but their role in culture mineralization remains equivocal.

In conclusion, the data presented here provide a detailed analysis of collagen synthesis and regulation in osteoblast cultures. These data indicate that collagen deposition is controlled at a posttranslational level and that normal collagen fibril formation and macrostructural assembly can occur in vitro. The nature of the mineral in these cultures is hydroxyapatite and it is associated primarily with collagen. Thus, osteoblasts in vitro offer an ideal system to study the temporal processes of collagen assembly and mineralization and to elaborate with further structural and biochemical characterization the possible roles of osteocalcin, phosphoproteins, and other extracellular components of the mineralizing matrix.

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