

Isolation of Bone Cell Clones with Differences in Growth, Hormone Responses, and Extracellular Matrix Production

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ABSTRACT Clones of nontransformed hormone-responsive bone cells have been isolated *in vitro* from mixed cell populations of fetal rat calvaria. In several independent isolations, microscopically visible colonies appeared at plating efficiencies of 5–10% of the starting cell numbers. Of these clones, ~10% grew to mass populations which could be assayed for a number of growth and biochemical properties. Although some similarities existed among the clones, they could be distinguished from each other and from the mixed cell populations. Population-doubling times (t_{DS}) and saturation densities varied over a wide range: e.g., t_{DS} of 24–72 h and saturation densities of $0.4\text{--}5 \times 10^5$ cells/cm². Morphologies varied from roughly polygonal multilayering cells to typically spindle-shaped monolayering cells. Hormone responsiveness, as measured by stimulation of cAMP by hormones, indicated that some clones were responsive to both parathyroid hormone (PTH) and prostaglandin E₂ (PGE₂), while others responded to PTH only. Analysis of extracellular matrix components revealed that all clones produced type I and type III collagens, though in different proportions. Similarly, although all clones synthesized four glycosaminoglycans (hyaluronic acid, heparan sulfate, chondroitin sulfate, and dermatan sulfate), the quantities of each were distinctive from clone to clone. Further investigation of such clones is continuing to define more precisely the heterogeneity of clonal bone cell populations *in vitro*. They represent an important step in the study of the endocrinology and differentiation of bone.

The study of hormonal control of bone cell metabolism has been advanced markedly by the culture *in vitro* of cells isolated from mouse and rat calvaria (21, 22, 24, 25, 28, 34). By sequential enzyme digestion, mixed populations of cells have been isolated which may be classified as osteoclastlike or osteoblastlike, based on a number of biochemical features (13). Initial characterization of these populations has provided evidence that both osteoclastlike and osteoblastlike populations respond to parathyroid hormone (PTH) while only the former respond to salmon calcitonin (sCT) (13, 34). However, studies of this type, which attempt to determine the molecular mechanisms of, for example, hormone desensitization (10) and hormonal regulation of collagen synthesis¹ (35, 36) are limited by several fundamental problems. First, populations of cells defined as osteoclastlike and osteoblastlike are, in fact, mixed cell populations. The degree of heterogeneity cannot adequately be defined in the experiments, i.e., fibroblastlike cells as well as

specific bone cells may be present in the populations. A more difficult to define functional heterogeneity may exist also in the possible presence of a variety of osteoclastlike or osteoblastlike cells in each population. For example, each population may consist of cells at various stages of differentiation or lifespan, and each class of such cells may have quite different hormone responsiveness. Second, it has been found that extended primary culturing or repeated subculturing of mixed bone cells results in changes of response, e.g., loss of PTH response (22) or change in ratio of type I to type III collagen (35, 36). Whether such changes reflect senescence of certain cells, differentiation *in vitro*, or overgrowth by some rapidly dividing subpopulation cannot be answered on such mixed populations. Third, determination of which particular cells in the mixed population are actually responsible for certain measured responses has not been possible. For example, whether or not some or all cells are responsive to PTH is not known, nor whether one sort of bone cell can make both type I and type III collagens.

These questions are best approached using a homogeneous

¹ Harper, R. P., J. N. M. Heersche, J. Sodek, and K. L. Yao. Collagen synthesis by isolated bone populations. Submitted for publication.

isogenic population. Such cells may be generated *in vitro* by cloning homogeneous populations from mixed cell populations. Clones of parathyroid hormone-responsive cells originating from nontransformed bone cell populations have not previously been described. We report here the isolation of clones of bone cells from mixed populations of early subcultures of rat calvaria. Preliminary characterization of the clones has included analyses of their growth properties, their responsiveness to certain hormones including parathyroid hormone, and their ability to synthesize specific collagen types and glycosaminoglycans.

MATERIALS AND METHODS

Isolation and Culture of Bone Cells

Bone cells were isolated from fetal rat calvaria by a modification of the methods of Wong and Cohn (36) and Rao et al. (25). 10 calvaria were minced a total of 20 times in spinner flasks containing 1 ml of PBS with 10 × antibiotics (1 mg/ml penicillin G, 500 µg/ml gentamicin, and 3 µg/ml amphotericin B). The PBS was removed and replaced with 1 ml of enzyme mixture (25). Incubation with continuous stirring was carried out in a 37°C water bath. After 10 min, the supernatant containing the cells was removed, filtered through a stainless steel sieve (200 mesh), and added to an equal volume of ice-cold fetal calf serum (FCS) to stop the enzyme activity. This was labeled population I (RCI). Fresh enzyme was added to the remaining bone chips and the digestion continued for a further 10 min (population II, RCII). A third 10-min digestion yielded population III (RCIII). Populations IV and V (RCIV and RCV, respectively) were harvested after two subsequent 20-min digestion periods. Populations isolated in this manner were centrifuged (10 min, 800 g), resuspended in α -MEM (Flow Laboratories, Inc., Rockville, Md.) plus 15% heat-inactivated FCS and antibiotics (as above at 1/10 stated concentration), and plated in 60-mm culture dishes at a concentration of 4×10^5 cells per plate. In some experiments, populations I and II were combined (designated population A, RCA) and populations IV and V were combined (designated population B, RCB). After 7 d of incubation at 37°C in 95% humidified air plus 5% CO₂, confluent cultures were washed once with phosphate-buffered saline (PBS) and the cells were detached by incubation with 3 ml of trypsin (0.25% in citrate saline) for 4 min. The enzyme activity was stopped by adding an equal volume of ice-cold FCS. Subculturing of mixed populations was performed routinely in this manner at ~1-wk intervals with seeding densities of $\sim 1.5 \times 10^4$ cells/cm². Colony-forming ability (plating efficiency) was determined by plating 10^2 – 10^3 cells on 60-mm culture dishes and scoring for macroscopically visible, stained colonies after 14 d at 37°C. The rat osteosarcoma cell clone, ROS 17/2, was given to us by G. Rodan (Department of Oral Biology, University of Connecticut) (see reference 14).

Cloning

Clones isolated from populations RCIII, RCIV, and RCB are reported in this paper (Fig. 1). The procedure in all cases was essentially as reported below for population III. Single-cell suspensions of 1st–4th subculture population III, prepared by trypsinization as described, were plated at limiting dilutions (e.g., ~1 cell/16-mm well) in 1 ml of α -MEM, 15% FCS, and antibiotics as described above. Trays were left undisturbed for 12–14 d, after which wells with microscopically detectable single colonies (0.5–2 mm diameter) were scored. Wells with more than one colony were recorded to determine plating efficiencies, but these colonies were not isolated or utilized further.

After identification of single-colony wells, old medium was aspirated and fresh medium added every 4–7 d, for 1–2 wk. Large colonies ($\geq 1,000$ cells) were subcultured; this was always well before confluence in the initial well since almost all clones tended to multilayer extensively and cells in heavily multilayered areas appeared to degenerate when left in this state. Subculturing was done by aspirating the medium, rinsing the well with PBS, and adding 2–3 drops of trypsin (as described above). As soon as the cells were rounded and easy to remove by gentle pipetting, 2 ml of fresh α -MEM + 15% FCS were added, and cells were resuspended and plated in fresh wells or 35-mm dishes. Thereafter, cells were subcultured on reaching confluence (though some areas could be multilayered; see Results). Cell seeding densities were normally kept at 1 – 2×10^4 cells/cm²; as populations attained higher numbers, they were maintained in T-75 flasks (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.), and some cells were frozen at -70°C to ensure stocks of clones.

Growth Curves

For growth curves, cells were plated in 60-mm culture dishes at desired seeding cell densities as given in Results. At various times usually starting 18 h after

plating, cells were trypsinized, resuspended, and counted in a hemocytometer. For such experiments over 2-wk intervals, medium was changed on all remaining dishes each 4–5 d.

Cumulative population-doubling levels (CPDL) were calculated according to Mueller et al. (18). Briefly, the number of population doublings (PD) that occurred in each subculture is given by: $\text{PD} = \log_2$ (cell density at subculture/[cell density at inoculation × attachment efficiency]). The CPDL is the sum of all PD throughout the lifetime of the culture. The CPDL for clones was estimated for the cloning procedure (e.g., 1 cell up to $\geq 1,000$ cells or $\text{CPDL} \geq 10$), this base level was added to CPDL accumulated for the mixed population before cloning, and those CPDL were more accurately determined for each clone as it grew to high cell numbers.

cAMP Responses to PTH, PGE₂, and sCT

Hormone incubations were carried out on confluent cell cultures in PBS, supplemented with 0.1% bovine serum albumin (BSA; Pentex [Miles Laboratories, Elkhart, Ind.]), 0.1% glucose, and 10 mM theophylline. 30 min before incubation, the culture medium was removed and replaced by 1 ml of supplemented PBS without theophylline. The cAMP incubation was initiated by the addition of an equal volume of supplemented PBS containing 20 mM theophylline, and either PTH (5 U/ml or as otherwise stated), prostaglandin E₂ (PGE₂, 5 µg/ml or as otherwise stated), sCT (400 mU/ml), or no hormone. Incubation was carried out at 37°C with air as the gas phase. Incubation times varied and are given with the respective tables and figures.

At the end of the incubation period, the medium was decanted and the cells were washed once for ~10 s with ice-cold PBS. After the PBS was decanted, the cell layer was extracted twice with 2×1 ml 90% propanol (2×24 h, at 4°C). The propanol extracts were pooled and the propanol was evaporated. cAMP in the dried extract was assayed according to Brown (2) as described previously (7).

Collagen Synthesis

To measure collagen synthesis, confluent cells were labeled with 2.5 µCi/ml [³H]proline (NEC-323; New England Nuclear, Boston, Mass.) in serum-free α -MEM supplemented with 50 µg/ml each of β -aminopropionitrile (β -APN) and ascorbic acid, and with proline adjusted to 3.5 mM.¹ A labeling period of 18 h was used, after which the medium was collected and heat-inactivated serum was added to a final concentration of 0.3% (vol/vol). The medium was exhaustively dialyzed against distilled water before equilibration with 0.05 M Tris-HCl buffer, pH 7.6. The collagenous proteins in a 0.5-ml sample were digested by the addition of 25 µl of a 1 mg/ml solution of bacterial collagenase (CPLSA; Worthington Biochemical Corp., Freehold, N. J.) purified as described by Peterkovsky and Diegelmann (23). *N*-Ethyl maleimide was added to a final concentration of 2.5 mM to inhibit residual protease activity, and the digestion was carried out at 37°C for 2 h. The digestion was terminated by the addition of trichloroacetic acid (TCA) to a final concentration of 7% (wt/vol). The precipitated proteins were pelleted by centrifugation at 10,000 g, washed in 1.0 ml of 7% (wt/vol) TCA, and repelleted. The supernatants were combined, the TCA was removed by ether extraction, and an aliquot was taken to measure the radioactivity associated with the degraded collagen. The pellet was dissolved in 100 µl of 70% formic acid, and an aliquot was taken to measure the radioactivity associated with the noncollagenous proteins. The percentage of radioactivity in the collagens was divided by 5.4 to compensate for the preferential incorporation of the [³H]proline into collagenous proteins (36). The cell layers were scraped off of the culture dishes after adding 2 ml of 7% TCA. The insoluble material was pelleted by centrifugation, washed until negligible radioactivity could be extracted, then heated to 90°C for 20 min to selectively solubilize the collagenous proteins.

To determine the nature of collagens synthesized, confluent cells were labeled with 1 µCi/ml each of [¹⁴C]glycine and [¹⁴C]proline (NEC-047H and NEC-285; New England Nuclear) in serum-free α -MEM deficient in these amino acids but supplemented with 50 µg/ml each of β -APN and ascorbic acid. After 18 h of labeling, the medium was removed and the cell layer was freeze-thawed three times and then extracted with 2 ml of 0.5 M acetic acid. Each fraction was exhaustively dialyzed against 1% acetic acid after the addition of serum to 0.125% (vol/vol). A sample of each was digested with 100 µg of pepsin in 0.5 N acetic acid for 4 h at 15°C. Both pepsin-digested and non-pepsin-digested samples were freeze-dried before analysis by SDS PAGE. The conditions used for electrophoresis and quantitation of radiolabeled collagen α chains have been described previously (11, 29). Identification of the various collagen α chains was made from comparisons of their mobility on electrophoresis gels in the presence and absence of mercaptoethanol with known standards.

Glycosaminoglycan Analysis

Confluent clones and ROS cells, cultured in 60-mm dishes, were labelled for 24 h with 200 µCi of [³H]acetate in 5 ml of α -MEM supplemented with 1% FCS

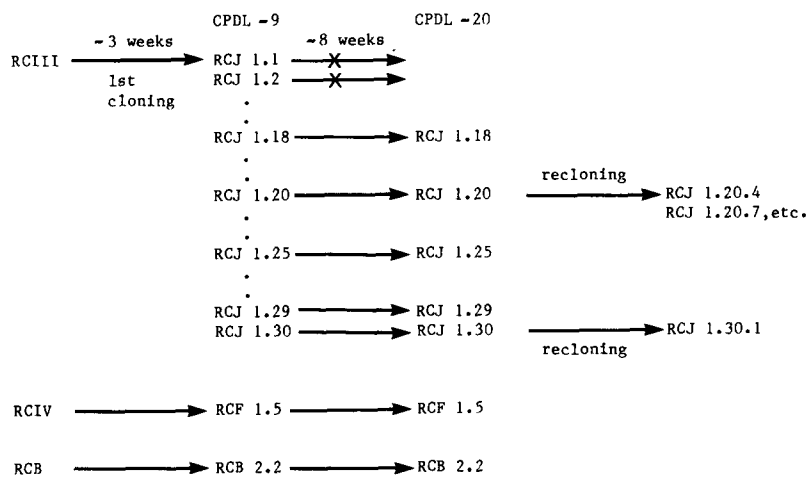


FIGURE 1 Isolation of bone cell clones. RCIII, RCIV, and mixed RCB populations were plated at limiting dilution in multiwell plating dishes as described. Many clones attained CPDL ≥ 9 ; a small percentage reached CPDL ≥ 20 (see text for details).

and the glycosaminoglycan (GAG) extracted from the growth medium and the cell layer separately, using the method of Niebes and Schifflers (19) as modified by Merrilees et al. (16). The growth medium (5 ml) or cell layer (5 ml of 0.01% trypsin in citrate saline) was digested in 25-ml polycarbonate centrifuge tubes with a 1-ml solution of protease (12.5 mg/ml; Type VI from *Streptomyces griseus*; Sigma Chemical Co., St. Louis, Mo.) in Tris buffer (pH 7.4) at 50°C for 16 h. After digestion, 3.3 ml of aqueous 31% NaCl and 0.18 ml of 3 N acetic acid were added before the samples were heated to 100°C for 5 min, cooled in an ice bath, and centrifuged at 30,000 g for 20 min at 4°C. The supernatant was transferred to 50-ml glass centrifuge tubes containing 21 ml of absolute ethanol and sodium acetate (0.8 g/liter) and left at 4°C for 72 h. Tubes were then centrifuged at 2,200 g for 20 min at 4°C. The supernatant was discarded and the precipitate taken up in 1–2 ml of distilled water and transferred to small test tubes for drying at 80–90°C. Each sample was redissolved in 40 μ l of distilled water, and a 1- μ l aliquot was electrophoresed on cellulose acetate membranes using a Beckman Microzone Cell (model R101; Beckman Instruments, Inc., Palo Alto, Calif.), 0.2 M zinc sulfate buffer, and a front current of 1 μ A/cm for 70 min (1). Membranes were stained for 8 min in 1% Alcian blue made up in a 1:1 ethanol:sodium acetate (4.1 g/liter) solution (pH 5.8) and destained by rinsing in an aqueous solution of 5% acetic acid and 10% ethanol. Membranes were then transferred to anhydrous methanol for 1 min and placed between two glass plates for drying.

GAG bands corresponding to hyaluronic acid (HA), dermatan sulfate (DS), and chondroitin sulfates (CS) 4 and 6 were identified using coelectrophoretic standards. Heparan sulfate (HS), for which no standard was available, was identified by its known position between HA and DS. Although the two CS could be clearly distinguished as standards, separation was less distinct in the samples, and for purposes of quantification these two GAG have been combined. The position of chondroitin 6-sulfate (CS6) is close to the known position of keratan sulfate (1); to check that this GAG was not present, selected samples of both the clones and the ROS cells were digested with chondroitinase ABC (aqueous sample digested with an equal volume of 0.2 M Tris buffer, pH 7.8, containing 0.02 U of enzyme/ μ l, for 16 h).

After identification, each band containing an individual GAG was cut out of the membrane, transferred to a scintillation vial, and digested for 20 min in 0.5 ml of 1,4-dioxan before adding 6 ml of scintillation fluid for counting.

RESULTS

Mixed populations RCIII, RCIV, and RCB, grew to high saturation densities in multilayered patterns. Colony-forming abilities of these populations measured at different times varied; normally, however, plating efficiencies of up to 25% were measured. A large variability in colony size was noted: i.e., about half of the colonies grew to diameters ≥ 2 mm ($\geq 1,000$ cells), whereas the rest grew to < 1 mm ($< 1,000$ cells) in 14 d. Such a heterogeneity was reflected also in the colony size resulting when clones were generated by limiting dilution plating in 16-mm cloning wells. Data from typical clonings of RCIII, RCIV, and RCB populations are shown in Table I. Depending on the starting population, the frequency of isolation of clones varied slightly, but actual percentages varied also within one population from one time of isolation to another. To ascertain whether the starting populations actually vary

TABLE I
Analysis of Numbers of Clones Attaining Measured Population-doubling Levels from Different Starting Mixed Populations

Starting population	Total no. of cells plated	No. of clones when screened*	No. of clones attaining $\geq 1,000$ cells, CPDL ≥ 9	No. of clones attaining $\geq 1-5 \times 10^4$ cells, CPDL $\geq 13-16$	No. of clones attaining $\geq 10^6$ cells, CPDL ≥ 20
RCIII	532	53 (10.4%)	30 (5.6%)	13 (2.4%)	5 (0.9%)
RCIV	360	31 (11.6%)	10 (2.8%)	3 (0.8%)	2 (0.5%)
RCB	1,152	26 (2.3%)	14 (1.2%)	4 (0.3%)	2 (0.2%)

* Clones were screened 2–3 wk after initial plating; all colony sizes were counted.

significantly in the percentage of clonable cells that each contains, many more isolations would have to be performed. In general, however, we have routinely isolated more clones from RCIII population than from other mixed populations.

A number of growth characteristics were determined for those clones considered most interesting in terms of initial screening assays of hormone responsiveness. Typical growth curves are presented in Fig. 2 for some of the clones soon after their isolation. It is evident that the RCIII mixed population was significantly different from some clones, e.g., RCJ 1.30 and RCJ 1.25, in terms of both doubling times (t_D) and saturation densities (Table II).

Some of the clones were re-cloned and tested also in several of the assays. Fig. 3 compares two RCJ 1.20 re-clones with the original clone; when tested, the t_D (~ 20 h, with saturation densities of $1-2 \times 10^5$ cells/cm²) were similar for all three.

As a comparison, the transformed rat osteosarcoma cell clone, ROS 17/2, was used in several assays. ROS 17/2 cells grew with a t_D of ~ 20 h, attaining saturation densities (in multilayered cultures) of $\sim 2 \times 10^5$ cells/cm².

Morphology

Figs. 4 and 5 summarize the morphological characteristics of the RCIII mixed population, compared with some of the clones, soon after initial cloning. In mixed population RCIII, areas of relatively polygonally shaped cells (Fig. 4a) were seen in the same flasks as areas with much more fibroblastlike cells (Fig. 4b). Cells in this RCIII mixed population multilayered extensively as did cells of many of the clones. Clones initially grew up usually as tightly packed multilayered colonies (data not

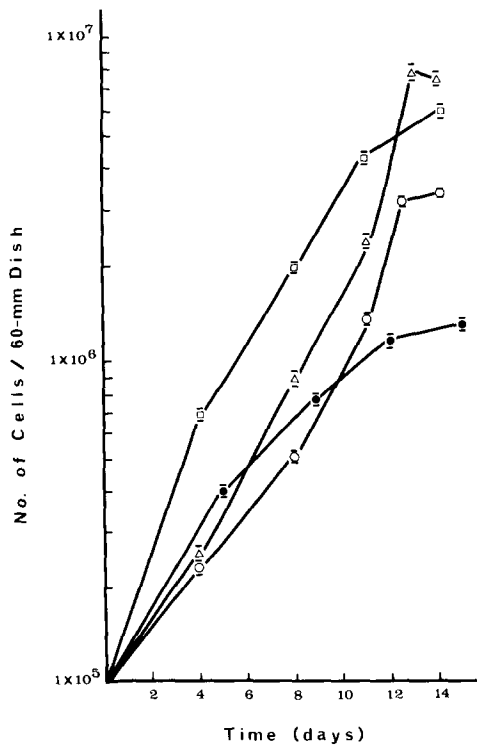


FIGURE 2 Growth curves for isolated bone cell clones. All clones were plated at the same starting cell densities; cell numbers were determined thereafter as total cells per 60-mm dish. Bars show the values determined from duplicate plates. (●) RCIII mixed population, (□) RCJ 1.20, (△) RCJ 1.30, (○) RCJ 1.25.

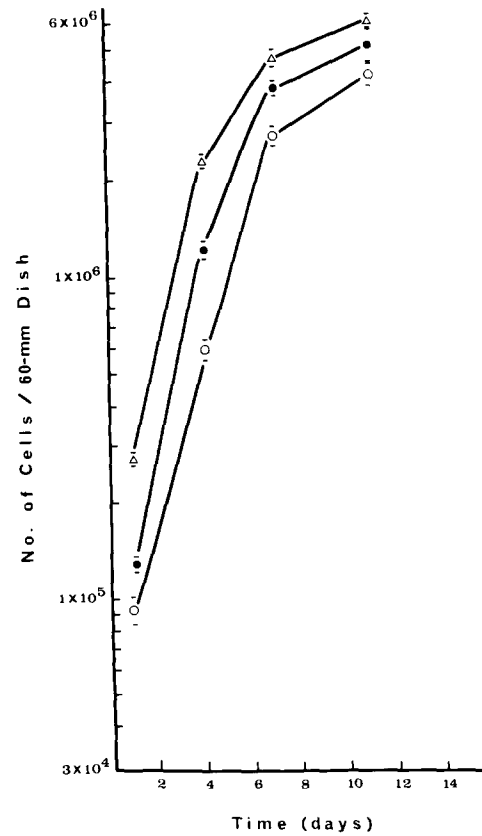


FIGURE 3 Growth curves for reclones of RCJ 1.20. Bars show the values determined from duplicate plates. (●) RCJ 1.20, (○) RCJ 1.20.7, (△) RCJ 1.20.2.

TABLE II
Growth Characteristics of Bone Cell Clones

Clone*	CPDL‡	t_D § h	Saturation density cells/cm ²
RCIII	20	62 ± 6	3.7 ± 0.4 × 10 ⁴
RCJ 1.20	44	38 ± 5	2.1 ± 0.3 × 10 ⁵
RCJ 1.30	38	53 ± 5	2.9 ± 0.3 × 10 ⁵
RCJ 1.25	40	72 ± 5	1.3 ± 0.3 × 10 ⁵
RCJ 1.29	57	48 ± 5	3.2 ± 0.4 × 10 ⁴
RCB 2.2	50	38 ± 10	1.0 ± 0.2 × 10 ⁵
RCF 1.5	50	48 ± 6	3.8 ± 0.2 × 10 ⁴
RCJ 1.20	50	24 ± 3	3.3 ± 0.2 × 10 ⁵
RCJ 1.30	50	29 ± 4	1.0 ± 0.1 × 10 ⁵
RCJ 1.20.7	53	24 ± 3	2.1 ± 0.2 × 10 ⁵
RCJ 1.20.2	53	24 ± 3	1.9 ± 0.2 × 10 ⁵
ROS	NA	20 ± 1	1.9 ± 0.2 × 10 ⁵

NA, not applicable.

* Origin of each clone is described in Fig. 1 and text.

‡ CPDL were calculated according to description in Materials and Methods and are approximate for cloned populations.

§ t_D is the population-doubling time measured from growth curves as shown in Fig. 2.

|| Saturation densities were calculated from growth curves as shown in Fig. 2.

shown). For one to two subcultures after isolation, almost all the clones grew quite slowly to monolayers of somewhat large, irregularly shaped or fibroblastlike cells (Fig. 4c) which multilayered in some areas. Cultures surviving this period normally grew somewhat faster and more uniformly thereafter. For example, soon after initial isolation and subculture, clone RCJ

1.29 (and several other clones not shown) always had areas heavily multilayered contiguous with monolayered, fibroblastlike cells (Fig. 4d); the cultures sometimes then became more uniformly multilayered (Fig. 4e). Occasionally, less heavily multilayered areas (arrows, Fig. 4e and f), sometimes occupied by large multinucleated cells, were evident. After two to three subcultures, clones assumed morphologies that were relatively stable and characteristic for long periods of time. Three clones for which this was true (RCJ 1.20, RCJ 1.30, and RCF 1.5) and which have been used extensively in subsequent studies are shown in Fig. 5. Clone RCJ 1.30 was made up of relatively flat, polygonal cells (Fig. 5a) which multilayered orthogonally (Fig. 5b). On the other hand, cells of clone RCJ 1.20 were somewhat more spindle-shaped, often displaying a raised, dark or refractile nuclear area (Fig. 5c), but also multilayered orthogonally (Fig. 5d). It is interesting to note that reclones of RCJ 1.20 were indistinguishable from the parent clone in morphology at low and high cell densities. Clone RCF 1.5 was the only clone isolated originally (although others have subsequently been isolated) with a very distinctive, more typically fibroblastlike, monolayering morphology (Fig. 5e and f). During initial testing, morphology seemed not to correlate with some other biochemical features of the cells (e.g., hormone responsiveness). Whether this will prove to be generally true for these cells in culture is not known (see below).

Hormone Responsiveness

Soon after initial isolation, to screen all clones including these which would not survive to high cell numbers, clones were subcultured equally into several 16-mm wells and allowed

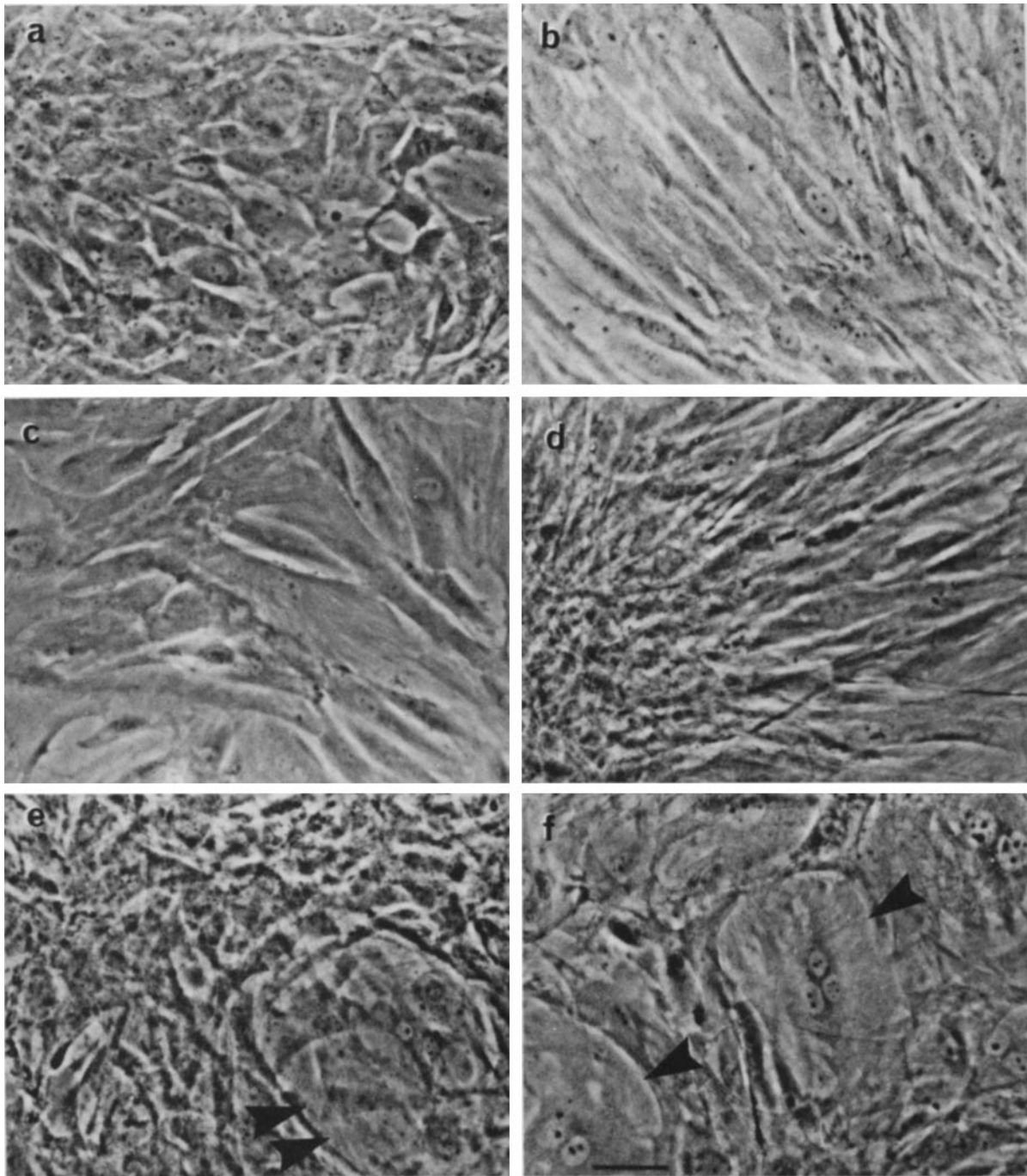


FIGURE 4 Phase-contrast micrographs of mixed population and clones of rat calvaria cells soon after isolation. RCIII mixed population contained areas of polygonally shaped cells (a) interspersed with more fibroblastic cells (b). After first subcloning, cells from almost all clones grew with quite flat, fibroblastic morphology (RCJ 1.25 is shown in c). RCJ 1.29 typically grew with heavily multilayered areas contiguous with monolayered fibroblastic areas (d); cultures of RCJ 1.29 left longer became more uniformly multilayered (e). Often, large multinucleated cells (arrows in e and f) appeared in these cultures; other cells did not grow over these. Bar, 20 μm . $\times 500$.

to grow to confluence. PTH stimulation of intracellular cAMP was determined for each clone (data not shown). Those responding above control levels were replated in 60-mm dishes and retested in a more complete assay with both PTH and PGE_2 . These latter data are summarized in Table III. Clones were not identical; rather, each clone was characterized by a particular hormone responsiveness. In this sort of analysis, clones fell into categories of being responsive to both hormones or to one only.

A more precise way of defining differences in hormone responsiveness is to analyze stimulation of cAMP with time of exposure to various hormones. This has been done for several of the clones, and a comparison of such data for two clones is shown in Fig. 6. RCJ 1.20 response to PTH clearly peaks at 2 min, whereas that of RCJ 1.30 peaks later at ~ 4 min, with a greater magnitude of response. cAMP was not stimulated in RCJ 1.30 by PGE_2 , whereas it was stimulated and peaked at 4 min for RCJ 1.20. Neither of these clones was responsive for

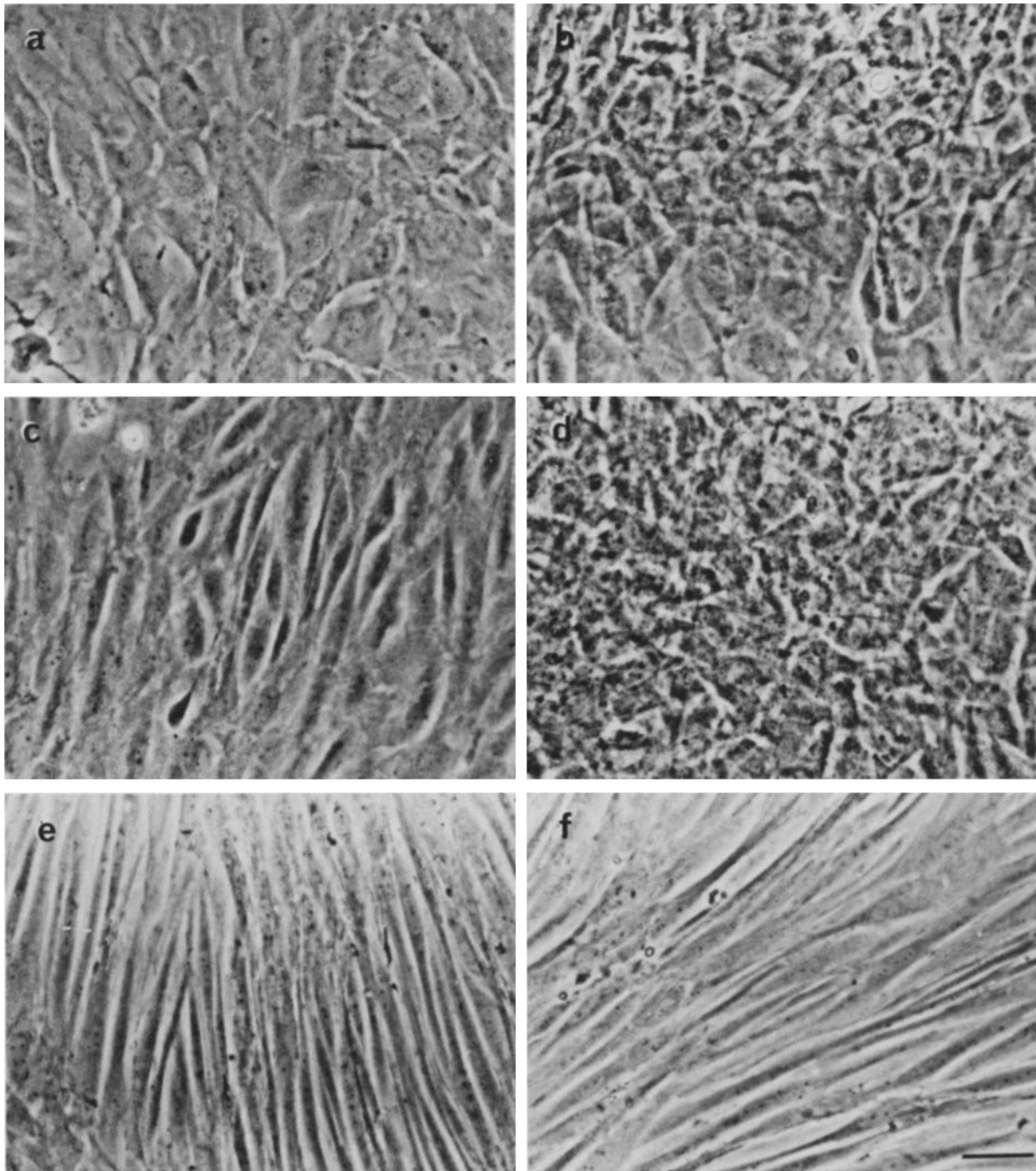


FIGURE 5 Morphology of bone cell clones. RCJ 1.30 at confluence appeared polygonally shaped with tight packing (a) and multilayering occurred orthogonally (b). RCJ 1.20 similarly multilayered orthogonally (d) but appeared more spindle-shaped in monolayer (c). RCF 1.5 had a very elongated spindle-shaped morphology with cells never multilayering (e and f). Bar, 20 μm . \times 500.

sCT. Table IV summarizes these and similar data for two other clones for which complete time-courses have been determined. These data were compared with those of ROS 17/2 cells, which were highly responsive to PTH (peak time, 4 min) and not responsive to PGE_2 (see Table IV).

Collagen Synthesis

The percentage of protein secreted by the various cells into the culture medium as collagen is shown in Table V. For the cloned cells the values varied, the lowest values being determined for RCJ 1.29 and the highest for RCJ 1.20. A similar

range of values was observed in the mixed bone cells in the RCA and RCB populations. However, the value for collagen synthesis by the ROS cells was significantly lower than the values for all but one (RCJ 1.29) of the cloned bone cells. Measurement of collagen in the various cell layers showed this to be <15% of the total collagen and did not account for the variability in the collagen analysis in the medium samples. However, ~45% of the total collagen was in the cell layer of ROS 17/2 cells.

Analysis of ^{14}C -labeled collagens by SDS PAGE revealed a similar pattern of procollagens and collagen α chains for the cloned cells, a representative scan of which is shown in Fig. 7.

Conversion of pro- α_1 and pro- α_2 chains to pc intermediates and to pn intermediates was observed with little conversion to α chains. In contrast to many other cell types, including the mixed bone cells, most clones revealed little or no labeled material in the position expected for fibronectin. After pepsin digestion, two major bands corresponding to the α_1 and α_2 chains of type I collagen and two minor bands migrating in the position of type III collagen α chains and the type V collagen α_1 chain were observed. Under nonreducing conditions, the type III band migrated in the position of collagen γ chains, whereas the other bands were unaffected. Quantitation of the radiolabeled α chains by densitometry demonstrated that, in the majority of clones, type III collagen comprised 1–2% of the total collagen and the $\alpha_1(V)$ chain <1%. Similar values were obtained with collagens synthesized by the ROS cells. However, appreciably more type III collagen was synthesized by RCB 2.2 and, in RCF 1.5, type III collagen comprised 14% of the total collagen synthesized. In the mixed population of bone cells, type III collagen synthesis varied between 1 and 20% of the total collagen synthesized and was dependent upon the density and time in culture.¹

GAG

Table VI summarizes the production of GAG by a variety of clones and ROS cells. All these bone cells tested synthesized four GAG (HA, HS, DS, and CS). While all the clones were similar in GAG profiles, each clone was distinct. Similarities, as indicated in Table VI, were as follows. First, the predominant GAG was HA, comprising 30–50% of the total; DS was

usually the next most prominent GAG. Second, a large amount (frequently >50%) of GAG was found in the cell layer. Third, it was notable that the proportion of HS was higher in the cell layer than in the growth medium; in the cell layer it frequently comprised >20% of the GAG. A number of distinctive differences should be pointed out for several of the clones. In clone RCJ 1.20, the proportion of HA was greater than in the other

TABLE IV
Hormone-induced Stimulation of cAMP

Clone	PTH (2.5 U/ml* or 1.25 U/ml‡)		PGE ₂ (2.5 μ g/ml or 1.25 μ g/ml‡)	
	Peak time min	cAMP pmol/60-mm dish	Peak time min	cAMP pmol/60-mm dish
RCJ 1.20‡	2	38.9 \pm 9.5	4	14.9 \pm 2.2
RCJ 1.30‡	4	63.3 \pm 8.6	4	2.2 \pm 0.4§
RCJ 1.29*	4	41.0 \pm 7.6	20	586.5 \pm 59.7
RCF 1.5*	4	52.7 \pm 9.4	4	252.2 \pm 37.0
ROS 17/2‡	4	33.9 \pm 3.4	20	11.6 \pm 1.6§

Times of peak stimulation and maximal value of cAMP in response to hormone were determined from complete time-course experiments as shown in Fig. 6.

* 2.5 μ g/ml or 2.5 U/ml.

‡ 1.25 μ g/ml or 1.25 U/ml.

§ Not significantly elevated from control values calculated on the same cells without hormone treatment.

TABLE V
Analysis of Collagen Synthesis by Bone Cell Clones

Cell type	Percent collagen synthesized %	Percent type III %	Percent $\alpha_1(V)$ %
Mixed populations			
RCA	13.3–14.3*	9–20	ND‡
RCB	12.2–15.4	1–14	ND
Clones			
RCJ 1.20	14.5 \pm 0.4	1–2	1
RCJ 1.25	13.0 \pm 0.2	1–2	1
RCJ 1.29	10.7 \pm 1.4	1–2	1
RCJ 1.30	13.4 \pm 0.6	1–2	1
RCJ 1.18	12.3 \pm 0.7	1–2	1
RCB 2.2	14.2 \pm 0.1	3–4	1
RCF 1.5	12.7 \pm 0.7	14	ND
ROS 17/2	10.5 \pm 0.1	1–2	1

* Mean of triplicates \pm standard deviation.

‡ ND, not determined.

TABLE III
Hormone Stimulation of cAMP in Bone Cell Clones

Clone	Effect of hormone treatment, cAMP pmol/60-mm dish		
	None (4, 10 min)	PTH (2.5 U/ml, 4 min)	PGE ₂ (2.5 μ g/ml, 10 min)
RCJ 1.18	1.5 \pm 0.1	13.9 \pm 9.5	5.4 \pm 1.8
RCJ 1.20	2.8 \pm 0.1	31.3 \pm 0.9	5.7 \pm 0.2
RCJ 1.25	2.5 \pm 0.2	25.9 \pm 6.3	9.2 \pm 0.9
RCJ 1.29*	4.0 \pm 0.5	45.6 \pm 2.9	58.3 \pm 3.5
RCJ 1.30	3.0 \pm 0.2	10.9 \pm 2.5	1.7 \pm 0
RCJ 1.20.2	2.2 \pm 0.2	15.6 \pm 0.2	10.1 \pm 2.7
RCJ 1.20.4	0.2 \pm 0.2	5.0 \pm 0.2	2.3 \pm 0
RCB 2.2*	7.4 \pm 1.0	105.6 \pm 9.8	5.5 \pm 0.7

Each value is the mean and mean deviation of duplicate determinations.

* Each value is the mean of triplicates \pm standard deviation.

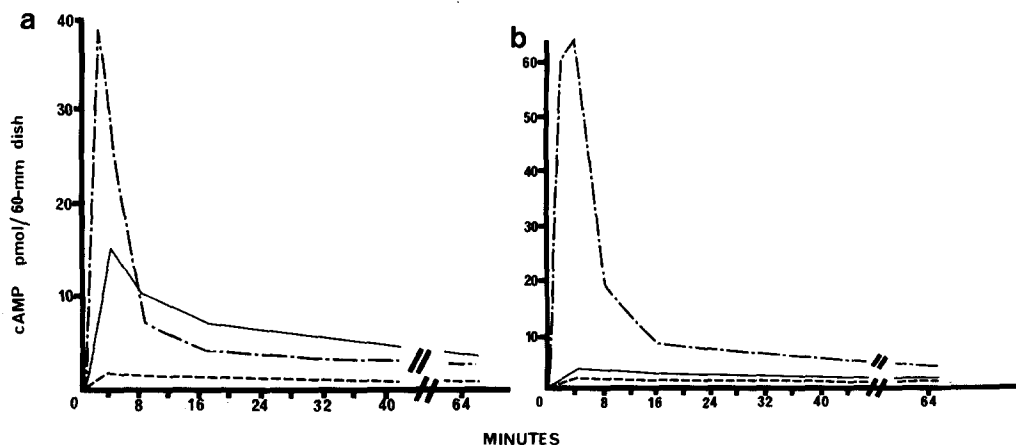


FIGURE 6 Time-course of hormone-induced cAMP response in bone cell clones. cAMP was measured at various times after addition of hormone. (a) RCJ 1.20 response to PTH (---), PGE₂ (—), sCT (---), or no addition (-.-). (b) RCJ 1.30 response to PTH (---), PGE₂ (—), sCT (---), or no addition (-.-).

clones. In fibroblasts (9) and smooth muscle cells (16), it has been found that the percent HA increases as cell density decreases. Clone RCJ 1.20 often attained higher densities than several of the other clones; comparison at equivalent cell densities showed that RCJ 1.20 still produced proportionately more HA. Note that reclone RCJ 1.20.4 had a profile similar

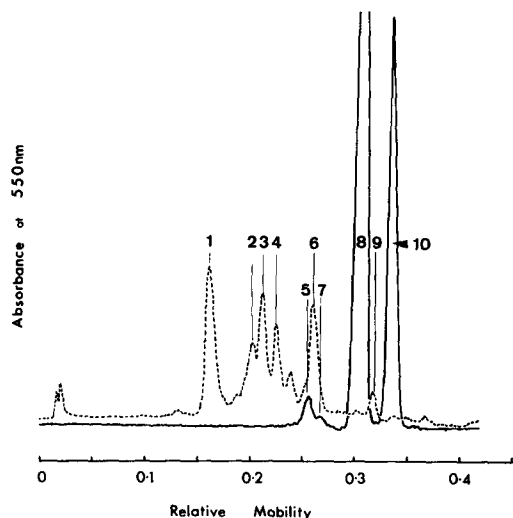


FIGURE 7 Spectrophotometric scans of ^{14}C -labeled collagens separated by SDS PAGE and visualized by fluorography. The scans were typical of the patterns obtained for the majority of clones. Procollagens evident in the non-pepsin-digested sample (----) were identified by comparison of mobilities with purified standards. Pepsin-digested sample (—) revealed α chains corresponding to type I, III, and V collagens. 1, pro- α_1 (I); 2, pc- α_1 (I); 3, pro- α_2 (I); 4, pc- α_2 (I); 5, α_1 (III); 6, pn- α_1 (I); 7, α_1 (V); 8, α_1 (I); 9, pn- α_2 (I); 10, α_2 (I).

to that of RCJ 1.20. Clone RCJ 1.30 and a reclone of it, RCJ 1.30.1, produced proportionately less HA and more sulfated GAG (both DS and CS) than, for example, clones RCJ 1.20 and RCJ 1.20.4. At comparable cell densities, less GAG was synthesized per cell in RCJ 1.30 than in other clones. RCJ 1.29 was high in HS, especially in the cell layer, compared to other clones. RCB 2.2 synthesized DS in large amounts relative to those of CS. RCF 1.5 was similar to RCB 2.2, but more HA was found, especially in the growth medium. In comparison, ROS cells differed from the clones in synthesizing predominantly CS rather than HA, and only a small amount of DS. Furthermore the CS corresponded in electrophoretic position to that of the CS6 standard rather than chondroitin 4-sulfate (CS4) as was the case for the clones. Chondroitinase ABC digestion confirmed that keratan sulfate, which has an electrophoretic position close to CS6, was not produced in either the clones or the ROS cells. As in the clones, the percent of GAG in the cell layer of ROS cells was high.

DISCUSSION

In this report, we have summarized the first isolation and initial characterization of clones of PTH-responsive cells from mixed bone cell populations. A number of features of these clones are of special interest: (a) Although clones of transformed osteoblastlike cells, rat osteosarcoma cells (e.g., ROS 17/2; see reference 14), and clones of nontransformed cells derived from bone but only responsive to PGE_2 (33) have been isolated, clones derived from nontransformed bone cells and responding to PTH have not been reported previously. The clones in this study were isolated at frequencies and with growth properties amenable with their being useful for biochemical and endocrinological studies. (b) The clones were heterogeneous in that

TABLE VI
Amount and Percentage of Labeled (^3H Acetate) GAG Synthesized by Confluent Clones and ROS Cells

Clone	Cells/ $\text{cm}^2 \times 10^{-6}$	Total GAG dpm/ 10^6 cells	Total GAG percent distribution				Growth medium GAG percent distribution				Percent GAG in cell layer	Cell-layer GAG percent distribution			
			HA	HS	DS	CS	HA	HS	DS	CS		HA	HS	DS	CS
RCJ 1.20	1.03 (0.35)	2,247 (290)	45.5 (2.1)	15.0 (1.4)	19.2 (0.1)	20.3 (1.4)	57.5 (2.1)	5.5 (2.1)	15.0 (1.4)	22.0 (2.8)	53.7 (2.1)	34.9 (1.4)	23.1 (2.8)	23.1 (1.4)	18.9 (0.1)
RCJ 1.20.4	0.77 (0.13)	2,072 (533)	49.7 (2.1)	17.3 (1.5)	20.7 (2.5)	12.3 (1.5)	58.7 (4.5)	5.7 (2.1)	20.3 (3.5)	15.3 (2.1)	56.5 (5.4)	42.6 (2.5)	26.4 (2.0)	20.7 (3.8)	10.3 (2.1)
RCJ 1.30	0.76 (0.25)	1,231 (154)	37.4 (1.7)	13.2 (1.0)	26.7 (1.2)	22.7 (0.6)	33.6 (2.1)	5.6 (0.6)	37.0 (5.8)	23.8 (4.0)	55.2 (2.5)	40.7 (5.5)	20.3 (2.1)	19.3 (1.5)	19.7 (6.9)
RCJ 1.30.1	0.36 (0.07)	2,620 (425)	39.6 (1.2)	9.3 (1.0)	23.1 (1.0)	28.0 (1.2)	37.8 (3.0)	4.3 (1.2)	25.7 (0.1)	32.2 (2.6)	36.9 (5.6)	41.9 (2.7)	17.7 (2.3)	18.9 (0.8)	21.5 (0.5)
RCJ 1.29	0.63 (0.11)	1,616 (352)	34.2 (2.6)	24.2 (0.6)	24.3 (2.3)	17.3 (2.3)	44.7 (4.2)	5.8 (0.8)	25.0 (1.7)	24.5 (4.6)	57.1 (4.2)	26.4 (7.5)	28.2 (3.6)	23.7 (4.0)	11.7 (2.3)
RCB 2.2	0.64 (0.11)	2,615 (790)	37.4 (0.6)	20.8 (0.6)	28.5 (1.5)	13.3 (1.0)	38.7 (1.2)	5.2 (1.5)	37.9 (1.5)	18.2 (1.0)	68.7 (1.5)	36.8 (0.6)	27.5 (1.0)	24.3 (2.1)	11.4 (1.5)
RCF 1.5	0.35 (0.06)	2,389 (390)	41.9 (2.0)	16.7 (1.1)	29.6 (0.5)	11.8 (0.9)	50.2 (3.2)	7.8 (2.0)	29.5 (2.0)	12.5 (1.1)	47.5 (4.2)	32.8 (3.8)	26.5 (2.2)	29.7 (1.6)	11.0 (1.1)
ROS	4.23 (0.4)	2,436 (66)	37.2 (0.6)	9.7 (0.6)	5.3 (0.6)	47.8 (1.0)	38.7 (1.2)	3.0 (1.0)	4.1 (0.4)	54.2 (1.2)	40.4 (6.0)	35.4 (0.6)	19.1 (0.5)	6.6 (1.2)	38.9 (0.2)

Each determination represents the mean of three samples with the standard deviation in parentheses. HA, hyaluronic acid; HS, heparan sulfate; DS, dermatan sulfate; CS, chondroitin sulfate 4 and 6 combined.

different clones had distinctive morphological and growth properties. Whether these will relate finally to functional capacities of the clones, reflecting also *in vivo* potentialities, awaits further isolation and characterization. (c) These clones have allowed for the first time an unambiguous analysis of hormone responsiveness in one clonally derived population of cells. Thus, for example, it is possible to state that: (1) cells derived from certain individual cells cloned from the calvarium can respond to both PTH and PGE₂, and (2) other cells can respond only to PTH. It is evident that both the magnitude and the peak time of response differ in different clones, though some clones may be similar to each other. It would be of interest to isolate and screen a variety of other clones to extend this catalogue of hormone responsiveness. (d) Individual clones of osteoblastlike cells were capable of synthesizing both type I and type III collagens. Previously such a conclusion has been postulated from some studies (26) but, as populations of cells in all these former studies were mixed, it was not possible to rule out the presence of a type III-producing contaminating subpopulation in the population of predominantly type I-producing cells.

The possibility that PTH and PGE₂ responsiveness might be associated with different cell types within osteoblastlike populations was suggested previously (8). In the experiments reported there, cAMP responses to PTH and to PGE₂ varied independently of each other when such populations were subcultured. The first unequivocal evidence that osteoblastlike cell populations responding to PTH but not to PGE₂ did exist was provided by the fact that ROS 17/2 cells responded to PTH and not to PGE₂ (G. Rodan, personal communication). However, since these cells represent transformed osteoblastlike cells, this observation might not necessarily imply that such cell types would exist in the nontransformed state. Unfortunately, it is not clear whether the nontransformed PTH-responsive and PTH- and PGE₂-responsive osteoblastlike cells isolated in the present communication are derived from and similar to osteoblasts, preosteoblasts, or undifferentiated osteoprogenitor cells. Further investigations of the metabolic properties of these cells in a variety of *in vitro* and *in vivo* systems are required to further our knowledge in this regard.

Since mineralized bone is generally considered to contain only type I collagen (17) and transformed bone cells have been reported to synthesize essentially only type I collagen (30), the synthesis of type III collagen by the cloned bone cells is, perhaps, surprising. However, it is apparent that most cells produced very small amounts of this collagen, and it is not known whether type III collagen is synthesized by bone cells *in vivo*. Immunohistochemical analyses have indicated that a small amount of type III collagen may be present in mineralized alveolar bone (31), but at these levels this would be difficult to determine biochemically.

Despite differences in the relative amounts of the interstitial collagens synthesized by the clones, the procollagen profiles were similar. A low amount of procollagen peptidase activity is often found for connective tissue cells in culture. However, the predominance of pn intermediates over pc intermediates is not usually observed. The apparent absence of fibronectin in radiolabeled culture media is also in contrast to most fibroblast systems. A similar observation has been made with mixed bone cells but only after an extended time in culture (6). The significance of this with respect to cell attachment by bone cells remains to be investigated.

Such a detailed analysis of GAG production in cloned or

mixed cultured bone cells has not been done previously. All clones isolated and analysed synthesized four GAG: HA, HS, DS, and CS (probably CS4). The predominant GAG associated with cortical bone *in vivo* appears to be CS4 (4, 15). Medullary bone, formed in the marrow cavity of bird long bones in response to estrogen, however, contains predominantly keratan sulfate (4). We are not aware of any detailed studies with regard to the GAG composition of osteoid or of woven bone, but it has been reported (32) that the sulfur content of osteoid decreases dramatically at the mineralization front in young rats. Of interest is the observation that GAG appear to be altered in osteogenesis imperfecta (3). A high percentage of the GAG in the bone cell clones reported here was found in the cell layer as compared with the growth medium. In contrast, in fibroblasts, usually <20% of the GAG is found in the cell layer, although in very dense cultures this percentage may increase. Clones with lower cell numbers had less material in the cell layer. This, combined with the extensive multilayering of the clones, suggests that considerable GAG may be built up in an extracellular matrix in the cell layer. The high proportion of HS in the cell layer as opposed to the growth medium is also consistent with a large amount of GAG in the cell layer and multilayering, since HS tends to be associated with the cell surface. It is notable, however, that clone RCF 1.5 also maintained a high percentage of GAG in the cell layer, although it had a strikingly fibroblast morphology and did not multilayer to any appreciable extent. This might argue that the GAG production by bone cells may be quite different from that of fibroblast-type cells. In this respect, it is interesting to note that while all the clones were broadly similar, each clone had a characteristic GAG profile.

What effect long-term culturing has had or will have on these bone cell clones is not yet known. All previous longer-term studies were carried out on mixed bone cell populations in which true genotypic and phenotypic alterations could not be separated unambiguously from outgrowth of certain subpopulations of cells. Preliminary data are available for a number of properties. After isolation, through at least 15 subcultures, the cells maintained approximately their growth properties. Gradually, these parameters altered (e.g., RCJ 1.20, RCJ 1.30, in Table I). If related to senescence as frequently reported for human diploid fibroblasts (6) and many other cells (for reviews, see references 5, 12, 18, 20, 27), then interestingly the CPDL for both these clones are now above 100, the *t_D* have not lengthened, and morphologically the cells do not display classical symptoms of senescence. Most of the clones (>80%) could not be propagated past CPDL of ~20 and may have been typically senescent. Since clones with long lifespans represented <1% of the starting cell population, they may comprise a small subpopulation of cells with much longer proliferative potential. An alternative hypothesis is that some of the clones may spontaneously transform in culture, as might be expected from their rodent origin. Further investigation of this point is ongoing. The fact that at least some characteristics ascribed to specialized bone cells survive in these cloned populations for long periods of time *in vitro* provides a new system for studying differentiated functions in this important cell type.

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Note Added in Proof: Further experiments have indicated that not only growth properties, but also hormone response, may change with extended time in culture. In addition, some clones acquire the property of tumorigenicity.

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