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Regulation of DNA Synthesis in Chick Calvaria Cells by Factors from Bone Organ Culture (41249)

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Abstract. Embryonic chick bones growing in organ culture release a substance into the culture medium which stimulates bone formation in previously untreated bones. This "conditioned" medium also enhances proliferation of monolayer cultures of chick calvaria cells in serum-free medium. The active principle is nondialyzable, indicating a molecular weight greater than 12,000 daltons. Dialysis also separates the mitogenic activity from a low-molecular-weight inhibitor. The amount of the mitogen found in conditioned medium increases as the rate of bone resorption increases in response to treatment with parathyroid hormone or 1,25-dihydroxyvitamin D_3. Maximal stimulation of DNA synthesis in calvaria cells is evident with conditioned medium obtained 3 to 5 days after treatment of bone cultures with parathyroid hormone. The cells must be treated with the conditioned medium continuously for 20 hr in order to obtain peak enhancement of DNA synthesis; there is no detectable effect in the first 8 hr. In contrast, the inhibitor acts within 4 hr. The data suggest that the stimulatory factor acts to increase cell proliferation by promoting entry of cells into the S phase of mitosis. We conclude that this stimulator is a locally produced regulator of bone formation, probably acting via an increased proliferation in osteoblast precursor cells.

Several lines of evidence from both clinical and animal studies have suggested that the processes of bone resorption and bone formation are coupled, such that a change in the rate of either process is compensated by a corresponding change in the other. This would prevent pathological loss or gain of bone mass. The existence of coupling was first suggested by the data of Harris and Heaney (1), who demonstrated a positive correlation between the rates of calcium removal and calcium accretion for the whole skeleton. Subsequently, several studies from this laboratory, using a rat model, have extended the coupling concept and indicated that it may occur under a wide variety of physiological and non-physiological conditions, including treatment with parathyroid extract (PTE), vitamin D, and diphosphonates, as well as in Ca or P deficiency (2–6).

Because systemic factors such as ions and hormones could not be invoked in the coupling mechanism, and because coupling is a localized, site-specific phenomenon (2, 3), recent work in our laboratory has focused on the concept that coupling is an expression of a local regulatory mechanism inherent in bone. In an attempt to demonstrate the coupling phenomenon in an *in vitro* system, Howard *et al.* (7) have shown that embryonic chick tibias cultured in a serum-free medium respond to acute PTE treatment with an increase in bone formation, in addition to the expected increase in bone resorption. The increase in formation occurred even after PTE was removed, and in the absence of other added hormones or growth factors. Furthermore, conditioned medium from resorbing bone cultures, which did not contain PTE, was found to stimulate OH-proline incorporation by previously untreated bones (8). This constituted our first direct evidence for a locally produced regulator of bone formation.

Further characterization of the active principle in conditioned medium was initially hindered by the amount of time re-

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quired to assay samples in the organ culture system, as well as the expense and labor involved in the dissection and culture of large numbers of embryonic tibias. We have thus attempted to assay the regulatory factor in cell monolayer cultures, using an osteoblast-rich cell population from chick calvaria. Since coupling *in vivo* is mediated by increases in both the cellular activity and the number of osteoblasts (2, 3), it was reasoned that the factor could be assayed by following its effects on either DNA synthesis in osteoblast precursors or collagen synthesis in mature osteoblasts. The present paper reports the results of experiments on the regulation of DNA synthesis in calvaria cells.

Materials and Methods. *Cell and organ culture.* Embryonic chick tibias and femora were isolated and cultured in serum-free BGJ_b medium (Fitton–Jackson modification, Gibco) as previously described (7). There were eight cultures per experimental group, with each culture containing two bones in 0.5 ml of medium. "Conditioned medium" was collected daily from both control (untreated) bone cultures and from cultures treated with 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) or PTE (10^{-9} *M* in each case). In the latter cases, the bones were treated for 24 hr, rinsed thoroughly to remove exogenous hormone, and placed in fresh medium without hormones. The conditioned medium (CM) was then collected at 24 hr intervals. Samples from each group were pooled and stored at 4° in glass vials.

Embryonic chick calvaria cells were isolated by collagenase digestion of the calvaria, using an adaptation of methods described for the isolation of fetal rat calvaria cells (9, 10). Briefly, the frontal and parietal bones of 17-day embryonic chicks were dissected and incubated at 37°, with shaking, in minimum essential medium (MEM) containing penicillin (100 units/ml), streptomycin (100 μg/ml), bovine serum albumin (1 mg/ml), and collagenase (2 mg/ml) (Worthington Type II) for 20 min. The cells released during this time were discarded and the incubation was continued for another 100 min. Collection and washing of the cells was as previously described (10). The cells were then suspended in BGJ_b medium and plated into plastic culture multiwells (16-mm diameter, Falcon), usually at a concentration of 250 cells/mm² in 1.0 ml (approximately 50,000 cells/well). Incubation was at 37° in 5% CO_2:95% air.

Dialysis of conditioned medium. Approximately 2 ml of fresh CM were transferred to a small-diameter dialysis tubing (Spectrapor 2, VWR Scientific) having a nominal molecular weight cutoff of 12,000 to 14,000 daltons. The tubing was placed in a screw-cap tube containing 2 ml of phosphate-buffered saline, pH 7.4 (PBS), and the tube was shaken gently at 4° for 12 hr. The sack was then transferred to a flask containing 1 liter of PBS, and dialysis was continued for 24 hr longer, with one additional change of buffer. The dialysate from the first dialysis (small volume) and the final retentate from large-volume dialysis were saved for testing in the cellular DNA synthesis assay.

DNA synthesis assay. Twenty-four-hour cell monolayer cultures were treated with CM (usually 50 μl/ml) for 18 hr, and [^{3}H] thymidine ([^{3}H]TdR) (40 Ci/mmole, New England Nuclear) was then added to a final concentration of 2 μCi/ml. After 2 hr of further incubation the medium was removed, and the cell monolayers were washed twice with 1 ml of MEM. The cells were removed by swabbing with a cotton-tipped applicator stick premoistened in 12.5% TCA. The cotton end was washed twice in 12.5% TCA (10 min each) and once in 95% ethanol (10 min). It was then dried, cut away from the wooden applicator, and placed directly into 5 ml of scintillation fluid (Aquasol I, New England Nuclear) for determination of radioactivity. This technique is a modification of that described by Gospodarowicz *et al.* (11), and in our experience the results are comparable in accuracy and reproducibility to those obtained by more conventional methods of cell harvesting, such as mechanical scraping or NaOH solubilization (12).

Alkaline phosphatase assay. Freshly isolated calvaria cells were pelleted at 800 *g*, washed twice in PBS, and resuspended in 0.1 *M* Tris–HCl, pH 8.3, containing 0.01 *M* sodium deoxycholate. They were ex-

tracted by shaking at 37° for 2 hr, followed by centrifugation to remove cellular debris. Aliquots of the supernatant were incubated at 37° with 2 ml of 0.01 *M* *p*-nitrophenyl phosphate in 0.045 *M* Na_2CO_3:0.005 *M* $NaBO_4$, pH 10.0. The reaction was stopped after 30 min by the addition of 1.0 ml of 0.5 *M* NaOH, and the OD at 410 nm was determined. One unit was defined as the amount of enzyme which results in a change in the OD_{410nm} of 1.0 under the specified conditions.

Results. *Characteristics of chick calvaria cells.* In order to test factors which could be involved in local regulation of either the proliferation or activity of bone-forming cells, it was desirable to obtain a population of cells rich in osteoblasts and their precursors which could be readily maintained in culture. Figure 1A demonstrates that the calvaria from 17-day-old chick embryos contain a high percentage of osteoblasts and their precursors, as indicated by the diffuse metachromatic staining and high alkaline phosphatase content which are known to be characteristics of osteoblasts *in vivo* (2, 6, 13). In contrast, very few osteoclasts (identified by staining for acid phosphatase, (6)) were evident. When the cells isolated from the calvaria by collagenase digestion were cultured in monolayers in serum-free BGJ_b, they maintained the gross morphology pictured in Fig. 1B, and they reached confluence in 4 to 6 days.

A large number of cells removed during the first 20 min of collagenase digestion were found to be red blood cells and superficial connective tissue cells. Furthermore, the extractable alkaline phosphatase in this population was low in comparison with that from cells released by further digestion (1.21 units/10^6 cells vs 3.83 units/10^6 cells). Thus, the cultures were enriched in cells with osteoblast characteristics by discarding this first population.

The pattern of DNA synthesis in the first 48 hr of culture is shown in Fig. 2. The rate of [^{3}H]TdR incorporation increases markedly within the first 24 hr after plating and declines sharply thereafter. However, the precise time at which incorporation becomes maximal is dependent on the density at which the cells are plated: if they are plated at 1000 cells/mm^2, the peak occurs at about 16 hr, but at the much lower density of 250 cells/mm^2 the peak occurs at about 24 hr. In subsequent experiments with CM, cultures plated at 250 cells/mm^2 were utilized, and test solutions were added 24 to 30 hr after plating. This procedure accomplishes two goals: (i) The time required for assay is minimized by waiting only 24 hr to begin the experiments; (ii) we avoid "masking" of mitogenic activities by the rapid cell division occurring immediately after cell isolation, while also minimizing the interference from an increasingly strong inhibition of DNA synthesis in denser cultures approaching confluence.

Effect of conditioned medium on DNA synthesis. In order to test the effects of CM

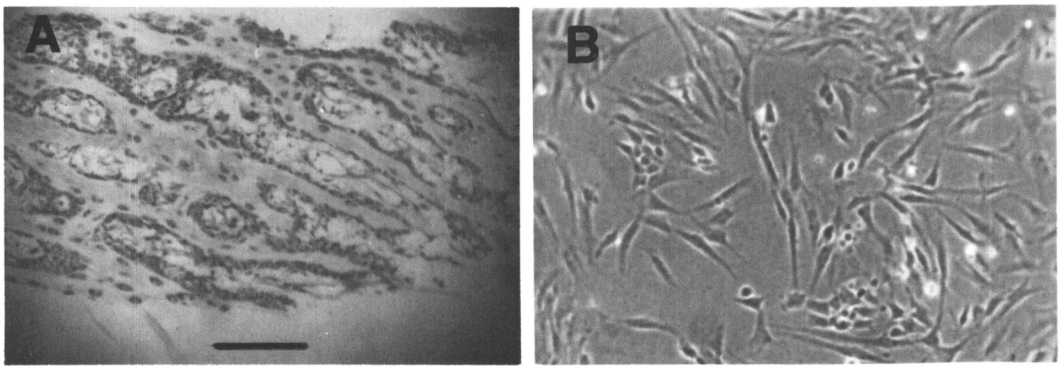

FIG. 1. (A) Transverse section through a 17-day embryonic chick calvarium. A 5-μm section was stained with methyl green and thionine. Bar = 100 μm. (B) Monolayer culture of cells isolated from 17-day embryonic chick calvaria, plated in BGJ_b, serum-free medium, 24 hr in culture. ×100.

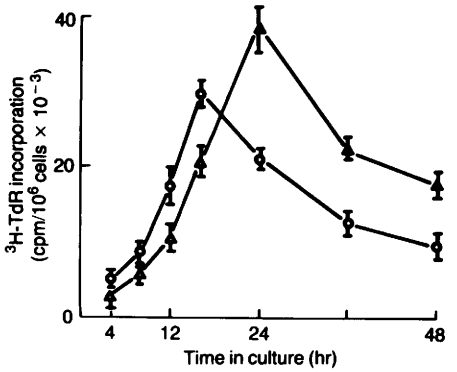

FIG. 2. [^{3}H]TdR incorporation into TCA-insoluble material as a function of time in culture at different plating densities. Circles, plating density = 1000/mm^2; triangles, plating density = 250/mm^2. Mean ± SEM, n = 6.

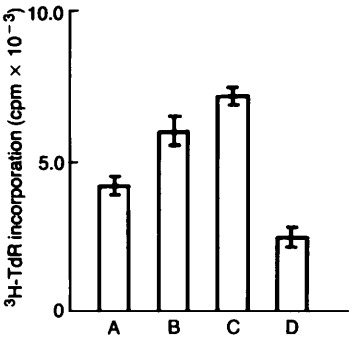

FIG. 3. The effect of dialysis on the activity of CM in the DNA synthesis assay. (A) Control (untreated cells); (B) undialyzed CM, 1:20 dilution; (C) retentate from dialysis of 5 ml of CM against 4 liters PBS; (D) dialysate from dialysis of 5 ml of CM against 5 ml PBS. Mean ± SEM, n = 6.

on DNA synthesis in calvaria cells, we employed samples of CM collected from bone cultures on the third day after PTE treatment (see Materials and Methods), since previous work had shown that these samples enhanced bone formation *in vitro* (8). Surprisingly, DNA synthesis in our cell assay was inhibited by 50 to 80%. It was subsequently found that freshly prepared CM gave a stimulatory response, but the stimulatory activity diminished with prolonged storage at 4°. Samples which had been stored for a month or more routinely inhibited thymidine incorporation. These results suggested that CM might contain both an inhibitor and a stimulator of cell proliferation, and that the latter substance was unstable during storage.

To test this hypothesis, we performed the experiment summarized in Fig. 3. CM was dialyzed against PBS in two stages: (i) Dialysis against an equal volume of PBS for 12 h; (ii) exhaustive dialysis against a large excess volume of PBS, with two changes. The dialysate from stage 1 and the retentate from stage 2 were then assayed for their effects on [^{3}H]TdR incorporation by calvaria cells. As shown in Fig. 2, the dialysate was inhibitory, while the retentate exhibited a stimulatory activity greater than that of the undialyzed CM. CM must therefore contain at least two factors regulating DNA synthesis: a stimulatory, high-molecular-weight factor (greater than 12,000 daltons, the nominal exclusion limit of the dialysis tubing) and a low-molecular-weight inhibitor.

Source of factors in conditioned medium. The rate of bone resorption in PTE-treated bone organ cultures is progressively higher, relative to the control rate, with increasing time after PTE treatment (7). If "coupled" bone formation is increased in proportion to the degree of resorption, as previously suggested (2, 7), and if the factor(s) in CM is involved in this process, then one might expect to find the highest mitogenic activity in CM collected several days after exposure of cultured bones to PTE. Figure 4 demonstrates that CM from PTE-treated bone organ cultures does influence DNA synthesis in calvaria cells, but the effect is dependent on the time at which CM is collected. Media collected on the first 2 days after removal of PTE either had no effect or were slightly inhibitory; interestingly, this inhibitory effect was more pronounced in CM from bones which had not been exposed to PTE. In contrast, the media collected in the next 3 days stimulated DNA synthesis, with a maximal increase of about 70% in cells treated with Day 5 medium. Some stimulation was also observed with media collected from control cultures, but the maximum was only about 20%.

The cultured bones were thoroughly washed after exposure to PTE, and it is thus highly unlikely that the observed

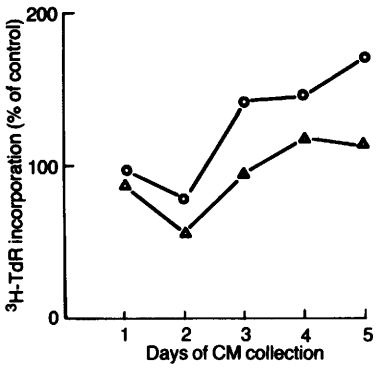

FIG. 4. Effect of CM from bone organ cultures (embryonic tibiae) on [^{3}H]TdR incorporation by calvaria cells. Circles, CM from PTE-treated bones; triangles, CM from untreated bones. All CM was added to cell cultures at a 1:20 dilution. $n = 6$.

stimulations of DNA synthesis by CM represent a response to residual PTE. To firmly establish this point, PTE was added directly to calvaria cell cultures in concentrations ranging from 10^{-12} to 10^{-6} *M* and [^{3}H]TdR incorporation was assayed as before. No concentration tested resulted in a statistically significant elevation of DNA synthesis, although at 10^{-6} *M* PTE the mean value was slightly higher (17% increase over control cells). Because of the known importance of 1,25-$(OH)_2D_3$ in bone metabolism (3), and also because of recent evidence that calvaria cells can produce this metabolite by hydroxylation of 25-$(OH)D_3$ (14), 1,25-$(OH)_2D_3$ was also tested over the range 10^{-12} to 10^{-6} *M*. Again, there was no significant effect on [^{3}H]TdR incorporation. The mean value at 10^{-6} *M* was 14% lower than control.

Howard *et al.* (7) have shown a positive correlation between the rate of resorption and the subsequent "coupled" increase in bone formation in cultured chick tibias. Together with the knowledge that the stimulatory substance in CM was not PTE or 1,25-$(OH)_2D_3$, these data suggested again that coupling could be a localized response to an increase in resorption. It was therefore of interest to know if CM from actively resorbing bones would stimulate DNA synthesis in calvaria cells regardless of the source of the resorptive stimulus. Table I demonstrates that treatment of the chick tibias in culture with either PTE or 1,25-$(OH)_2D_3$ results in an increased rate of resorption, and the CM from either group causes significant stimulation of DNA synthesis in calvaria cells.

Timing of the response to CM. When stimulatory CM was added to 24-hr cell cultures and DNA synthesis was assayed at 4-hr intervals, there was no detectable effect within the first 12 hr, but there was an increasing enhancement of DNA synthesis between 12 and 24 hr (Fig. 5). In contrast, inhibitory CM causes a depression in DNA synthesis within the first 4 hr, and there is actually some recovery from inhibition after 8 hr. Thus, peak *inhibition* is apparent at about 4 hr after treatment, whereas peak *stimulation* is evident at about 20 hr. In addition to establishing optimal assay times, this experiment also suggests very different sites of action for the factors in CM.

TABLE I. THE EFFECT OF PTE AND 1,25-$(OH)_2D_3$ ON THE RELEASE OF OH-[^{3}H]PROLINE FROM PRELABELED BONES AND ON THE PRODUCTION OF STIMULATORY CM

Treatment	OH-[^{3}H]Proline released (cpm/ml)	cpm [^{3}H]TdR in calvaria cell DNA
10^{-9} *M* PTE	427 ± 40[a]	4872 ± 361[b]
10^{-9} *M* 1,25-$(OH)_2D_3$	411 ± 31[a]	4963 ± 289[b]
Control	242 ± 36	3141 ± 207

Note. Bone were prelabeled for 24 hr with [^{3}H]proline and then treated with either PTE or 1,25-$(OH)_2D_3$ for an additional 24 hr. Total OH-[^{3}H]proline release into the medium was determined as previously described (7). For production of CM, the same experiment was performed on unlabeled bones, and CM was collected as described under Materials and Methods. Day 3 CM was used in the DNA synthesis assay at a 1:20 dilution.

Mean ± SEM, $n = 6$.

[a] $P < 0.005$.

[b] $P < 0.001$.

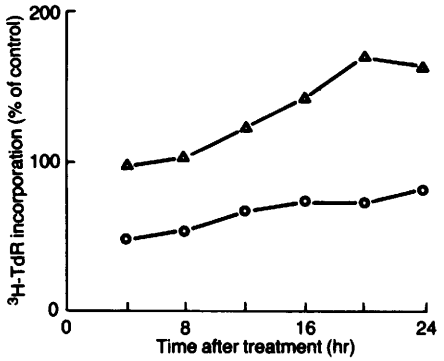

FIG. 5. Incorporation of [^{3}H]TdR by calvaria cells as a function of time after treatment with stimulatory or inhibitory CM. Triangles, stimulatory CM from Day 5, PTE-treated bones; circles, inhibitory CM from Day 2, untreated bones. CM was added at a 1:20 dilution to 24-hr cell cultures. $n = 6$.

Discussion. The development of an assay system utilizing chick calvaria cells grown in a serum-free medium has provided a convenient and rapid assay for further characterization of regulatory factors produced by bones in organ culture. Rat calvaria have been used extensively as a source of bone cells for *in vitro* experimentation (9, 10, 15, 16), and fractionation of these cells together with histological examination has shown that there is a large number of osteoblasts and their progenitors in calvaria, with very few osteoclasts (10). The morphology of the chick calvaria cells and their high content of alkaline phosphatase indicate that they also contain a high percentage of osteoblasts and thus constitute a convenient model system for the assay of factors regulating bone formation.

We have shown that isolation of calvaria cells by digestion with bacterial collagenase induces a period of rapid cell division in the first 24 hr of culture, as evidenced by the marked increase in [^{3}H]TdR incorporation into DNA. Incorporation peaks earlier and declines more rapidly in cells plated at high densities, which clearly indicates a normal inhibition of cellular proliferation as the cells approach confluence. The source of this inhibition is unknown, although experiments with 3T3 cells suggest that diffusible inhibitors of proliferation accumulate in dense cultures (17). Alternatively, reduced cell division could be a consequence of contact inhibition mediated at the cell surface, or of degradation of a naturally produced mitogen.

CM from actively resorbing bone organ cultures causes increased bone formation in previously untreated bones (8), and it was reasoned that this could result from an increase in cell division in osteoblast progenitors, leading ultimately to an increase in the total number of mature osteoblasts. However, when testing this hypothesis in the calvaria cell assay system, it was found that there are at least two regulatory factors present: a high-molecular-weight stimulator of DNA synthesis, and a low-molecular-weight inhibitor. This result, though surprising, is not without precedent. For example, cultures of macrophages from rat marrow produce both an inhibitor and a stimulator of cell proliferation, and these factors were separable by dialysis (18). The presence of apparently competing factors in the same tissue or cell population may represent a regulatory system of checks and balances on cellular activities. At any rate, when both inhibitors and stimulators of proliferation are present in the same culture, as in those treated with undialyzed CM, they would be expected to counteract each other, and the net effect on DNA synthesis must be at least partially dependent on the relative concentrations of these factors.

The stimulations of DNA synthesis reported in this paper are comparatively small, and it is not clear whether this is due to the presence of only very low concentrations of mitogen in CM. As indicated in Fig. 2, DNA synthesis normally shows a progressive decline as the cultures approach confluence, and it is possible that the mitogen concentrations are so low that they can not greatly counteract the normal inhibition of cell division, even in the less dense cultures used in this work. The small stimulation cannot simply be due to a generalized diminution in cellular responsiveness to mitogens, since we have observed increases in [^{3}H]TdR incorporation of up to 600% in cultures treated with 5% fetal calf serum. However, the serum response is a relatively nonspecific effect, and if the

target cells for the factor in CM constitute a very small percentage of the total cells, then the stimulations observed here could be the maximum obtainable. If this is not true, and if the mitogen concentrations are well below the optimum, then suitable methods for concentrating the test samples of CM should increase the effect. This possibility is currently under investigation.

The timing of the response to stimulatory CM is of particular interest in regard to the coupling hypothesis, since the minimum 12-hr delay between treatment and response indicates that the mitogen acts by promoting entry of cells into the S phase of mitosis, rather than by acceleration of [^{3}H]TdR incorporation into cells already engaged in DNA synthesis (19). This is consistent with the observations of Owen (20), who showed by autoradiography that episodes of resorption *in vivo* are followed by increases in cell division in osteoblast progenitors, resulting ultimately in the movement of new, mature osteoblasts to the bone surface. It has been pointed out that compensatory, coupled bone formation *in vivo* would take 1–3 days if production of new cells is required, and this delay is observed in many experimental systems (2, 7, 20).

The enhancement of DNA synthesis diminishes after the first 24 hr of treatment with CM, and the rate returns to control levels by 48 hr. It thus seems probable that the factor is either degraded after an initial response, or the cells in culture become refractory after one exposure. In either case, the phenomenon raises interesting possibilities for further regulation of bone formation.

In contrast to the stimulatory factor, the inhibitor acts very rapidly, suggesting that it could be interfering directly with the machinery of DNA synthesis. This factor is dealt with more extensively in a separate paper (12) and is not considered further here.

Baylink and Liu (2) have argued extensively that bone has the means to regulate itself at the local level, and the production of regulatory factors by bone organ cultures strongly supports this hypothesis. In addition, it has been shown that the effects on isolated cells are probably not due to residual contamination of conditioned medium by 1,25-$(OH)_2D_3$ or PTE. However, the precise source of the mitogen in CM remains unknown. Because during coupling the increase in bone formation is proportional to the increase in resorption, it is logical to suppose that the factor is either produced by osteoclasts or else released from bone matrix by osteoclastic activity. In our own work with cultured osteoclasts from chicken and rat long bones, we have been unable to demonstrate production of either mitogenic or inhibitory factors. However, if *osteoblasts* produced and secreted the stimulatory factor along with matrix components, it would eventually become an integral part of matrix. Presumably it would be released during resorptive episodes and proceed to initiate mitosis in osteoblast precursors. The early work of Urist *et al.* (21) indicated that such a substance does exist in matrix, and subsequent work has shown that it can be extracted from bone by mixed aqueous–nonaqueous solvents (22) or by the use of strong guanidinium–HCl solutions (23). Of particular interest is the demonstration of large amounts of this protein in osteosarcoma tissue, in which there is a rapid proliferation of osteoblasts (24, 25). More recently, Peck *et al.* (26) have shown that cultured rat calvaria cells produce a factor which stimulates DNA synthesis when added to quiescent cultures of these same cells.

In conclusion, this work has demonstrated the existence of a high-molecular-weight factor which enhances DNA synthesis in calvaria cell cultures, apparently by promoting entry of quiescent cells into a cycle of cell division. The mode of production of this factor and its action in the isolated cells are consistent with a role in coupling between bone resorption and formation. However, substantiation of this theory will require development of a means to quantitate factor production and to unequivocally determine its source.

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