

An Immortalized Osteogenic Cell Line Derived from Mouse Teratocarcinoma Is Able to Mineralize In Vivo and In Vitro

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Abstract. The hybrid plasmid pK4 containing the early genes of the simian virus SV-40, under the control of the adenovirus type 5 Ela promoter, was introduced into the multipotent embryonal carcinoma (EC) 1003. Expression of the SV-40 oncogenes was observed at the EC cell stage, and this allowed the derivation of immortalized cells corresponding to early stages of differentiation.

Among the immortalized mesodermal derivatives obtained, one clone, C1, is committed to the osteogenic pathway. C1 cells have a stable phenotype, synthesize type I collagen, and express alkaline phosphatase activity. Although immortalized and expressing the SV-40

T antigen, the cells continue to be able to differentiate in vivo and in vitro. In vivo, after injection into syngeneic mice, they produce osteosarcomas. In vitro, the cells form nodules and deposit a collagenous matrix that mineralizes, going to hydroxyapatite crystal formation, in the presence of β -glycerophosphate. This clonal cell line, which originates from an embryonal carcinoma, therefore differentiates into osteogenic cells in vivo and in vitro.

This immortalized cell line will be useful in identifying specific molecular markers of the osteogenic pathway, to investigate gene regulation during osteogenesis and to study the ontogeny of osteoblasts.

ONE current view of the development of bone cells is that osteocytes derive from primitive mesoblastic cell precursors through a cascade of events. These involve, in the case of intramembranous ossification, (a) proliferation of primitive mesoblasts, (b) differentiation of these cells into an osteoprogenitor cell, then into osteoblasts, and (c) maturation of osteoblasts with synthesis of a collagen matrix and mineralization (5, 10, 39, 44). Up until now, models for following bone differentiation in vitro have been of two types: (a) osteoblast-like clones explanted from normal bone, and (b) clones derived from osteogenic osteosarcomas.

(a) The former are normal explanted cells capable of progressively synthesizing a bone-like tissue in the presence of organic phosphate and ascorbic acid (1, 3, 8, 9, 28, 35, 46). This process and its hormonal regulation can therefore be studied in vitro. Collagen I maturation, in particular, has been extensively studied (11). However, the potential for division of such cells remains limited, and this precludes molecular studies. In addition, phenotypic changes with loss of osteoblastic properties often occurs in culture (47).

(b) Clones derived from rat osteosarcomas have also been useful in investigating the effects of hormones (parathyroid hormone; prostaglandins) and vitamins (retinoids and vitamin D3) on bone development (17, 25, 29, 30, 41). The formation of a mineralized matrix has, however, been shown to require either the implantation of the tumoral cells in diffusion chambers within the animal (43), or the growth of the cells in agar (37).

In both model systems, the major problems encountered are the heterogeneity of the cell population within a clone, and the lack of specific markers allowing formal identification of the progressive stages of osteoblast maturation.

In this study, we introduce mouse teratocarcinoma as a new model for studying bone differentiation. This system has already proven useful in the study of determination and differentiation mechanisms in early embryogenesis. Teratocarcinoma tumors contain a wide variety of differentiated tissues derived from a multipotent stem cell, the embryonal carcinoma (EC)¹ (19). The differentiated derivatives, however, have a limited potential for division, preventing the isolation of pluripotent progenitors committed to the various restricted cell lineages. We have recently developed a strategy for immortalizing such stem cells from teratocarcinomas. A recombinant plasmid, pK4, was constructed, in which the SV-40 oncogenes were linked to the adenovirus 5 Ela promoter. This promoter is active in early embryonic cells (6). In a first series of experiments, the plasmid was introduced into the EC F9 (20, 22). Immortalized cells were obtained after retinoic acid induction. From these cells, precursors of different lineages could be selected and cloned (21). Osteogenic cells were not found among the mesodermal derivatives.

In a series of parallel experiments, pK4 was introduced into 1003 (31), a multipotent EC cell line known to undergo

1. Abbreviation used in this paper: EC, embryonal carcinoma.

mesodermal differentiation with high efficiency (7). We show in this paper that expression of the SV-40 oncogenes was observed in 1003-pK4 cells as early as the EC cell stage. Expression did not prevent further differentiation because the tumors obtained after injection to syngeneic animals contained differentiated cells representative of the three embryonic germ layers: neuroectoderm, endoderm, and mesoderm. Among the immortalized mesodermal derivatives, we were able to select, on the basis of its high expression of the bone-specific alkaline phosphatase, a clone, C1, committed to the osteogenic pathway. C1 maintains a stable phenotype during exponential growth. Upon differentiation *in vivo*, it gives rise to typical osteosarcomas. In the presence of appropriate inducers, it mineralizes *in vitro*, forming dense calcified nodules.

Material and Methods

Cell Culture and Differentiation Conditions

1003 cells (7) and their derivatives were routinely grown in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (Gibco Laboratories).

Cloning by limiting dilution (0.3 cells per 6-mm microtiter wells) was performed in multidishes (Costar Corp., Cambridge, MA). Clones 1003 transformed by pK4 were obtained after cotransfection of the embryonal carcinoma 1003 with plasmids pK4 and PSVtkneo β using the calcium phosphate precipitation technique as described (21). An intense staining of >10% of the cells with anti-SV-40 T antibodies was detected 48 h after the transfection. Colonies resistant to Geneticin (Sigma Chemical Co., St. Louis, MO, 400 μ g/ml) were isolated and subcloned.

C₁ cells were routinely grown on tissue-culture dishes in DME supplemented with 10% FBS.

To induce *in vitro* mineralization, C1 cells were first seeded on untreated plastic dishes (Sterilin, Ltd., Hounslow, UK). After 8 or 15 d, the three dimensional clusters formed were either plated onto tissue-culture dishes on which they reattached, or maintained as aggregates. After 2 d, the medium was shifted to DME supplemented with 1% FBS, and some dishes were treated with ascorbic acid (50 μ g/ml, Sigma Chemical Co., St. Louis, MO) and β -glycerophosphate (7 mM, Sigma Chemical Co.). The nodules, which developed rapidly, were fixed for light or electron microscopy.

Tumor Formation

2–4 $\times 10^6$ cells in 1 ml DME were injected subcutaneously to irradiated (600 rad) C₃H syngeneic mice. One part of the 1003-pK4-derived tumors was fixed in Bouin's fluid and processed for routine histological analysis. Another part of the tumor was minced and allowed to grow and differentiate *in vitro*.

C1-derived tumors were fixed in 2% glutaraldehyde, dehydrated in alcohol and embedded undecalcified in methacrylate (2, 16). Thick sections (5 μ m) were cut according to conventional methods (2).

Southern Blot Hybridization of Genomic DNA

Aliquots (10 μ g) of genomic and pK4 DNAs were digested by restriction endonucleases, electrophoresed on a 0.8% agarose gel and blot-transferred using zeta-bind membranes (Cuno, Inc., Meriden, CT). Hybridization was carried out using a pK4 probe nick-translated using an Amersham kit (Amersham International, Amersham, UK) (3×10^8 cpm/ μ g).

Immunocytochemistry

Indirect immunofluorescence studies on cells were carried out using several antisera: (a) mouse mAb Pab 419 (32) detecting viral T antigens and mouse mAb ECMA 7 directed against specific EC cell-surface antigens (23); (b) affinity-purified rabbit antibodies raised against basal membrane components: collagen I and III (14). (c) species-specific secondary antisera coupled to fluorophores (Cappel Laboratories, Malvern, PA) were used to visualize sites of primary antibody binding.

Fixation and permeabilization of the cells are described in the figure legends.

Controls consisted of cells incubated without primary antibody or with nonimmune serum.

Biochemical Measurements of cAMP

The response of C1 cells to 1-34 human parathyroid hormone (PTH; Sigma Chemical Co.), prostaglandin E₂ (PGE₂; Sigma Chemical Co.) and salmon calcitonin (CT; kindly provided by Rorer Laboratories, Gennevilliers, France) was evaluated by determination of the intracellular production of cAMP. Confluent cells were treated for 10 min with PTH or the solvent (PBS), PGE₂ or the solvent (0.1% ethanol) or CT, or the solvent (PBS). Intracellular cAMP concentrations were determined in cell lysates using a specific RIA (Immunotech, Luminy, France).

Biochemical Measurements of Alkaline Phosphatase Activity

This was carried out according to previously reported methods (28). The thermosensitivity of the enzyme was checked according to Goldstein et al. (12).

Histochemical Identification of Alkaline and Acid Phosphatase Activities

Confluent cells and nodules were fixed at 4°C in 95% ethanol. Cells and nodule sections were exposed to naphthol ASBI phosphate (30 min, 37°C, pH 8.6) and naphthol ASTR (Sigma Chemical Co.) (1 h, 37°C, pH 6), used as substrates for alkaline and acid phosphatase activities, respectively (28). The cells were observed with or without counterstaining with hematoxylin.

Collagen Analysis

Confluent cells were labeled for 24 h with 50 μ Ci/ml of 4.5 [³H]proline (32 Ci/mmol; Commissariat à l'Energie Atomique, Saclay, France) in DME supplemented with 1% FBS, 100 μ g/ml ascorbic acid, and 50 μ g/ml *b*-amino-propionitrile fumarate (Sigma Chemical Co.). The cells were collected in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 25 mM EDTA, 10 mM *N*-ethylmaleimide, and 2 mM PMSF, and pooled with the culture medium. After sonication and addition of TCA (10%), the insoluble residue was collected by centrifugation.

The amount of [³H]proline incorporated into collagenase-digestible protein and noncollagenous protein was determined as described (40).

Collagen types were determined by PAGE: pellets resuspended in 0.5 M acetic acid were digested with 100 μ g/ml pepsin (Sigma Chemical Co.), pH 2.0, for 4 h at 4°C. The pepsin digest brought to pH 8.0 was dialyzed and lyophilized. Samples were then analyzed by SDS-PAGE. Delayed reduction with 2-mercaptoethanol was used in some samples. Labeled proteins were visualized by fluorography.

Histological Detection of Collagen and Minerals

After 15 d of culture in the presence or absence of ascorbic acid and β -glycerophosphate, the nodules formed were collected, fixed in 95% ethanol, and embedded in glycolmethacrylate. 5- μ m-thick sections were stained with toluidine blue, Von Kossa, or Goldner Trichrome for the detection of collagen and minerals (2, 28).

Electron Microscopy, X-Ray Microanalysis, and X-Ray Diffraction

The calcified nodules formed in differentiated C1 cultures were fixed *in situ* with 2% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2, 20 min). The nodules were post-fixed in 1% osmium tetroxide (15 min), rinsed, dehydrated in acetone, and embedded in Epon. Ultra-thin sections stained with 2.5% uranyl-acetate and lead citrate were examined using a Siemens Elmiskop 101 electron microscope (Siemens-Allis, Inc., Cherry Hill, NJ). Mineralized spots were analyzed on carbon-coated sections using energy dispersive x-ray microanalysis (Tracor Northern, Middleton, WI). X-ray diffraction analysis of the calcifying structure was performed and calibrated using an aluminum standard.

Results

Selection and Cloning of an Immortalized Osteogenic Clone from pK4-transformed Teratocarcinoma

Expression of the *pk4* Genome at the EC Cell Stage in Transformed 1003-pK4 Clones. 1003 EC cells were cotransfected with plasmids pK4 and PSVtk-neo β . Geneticin resistant clones were then selected. Out of 15 clones analyzed, 3 (clones 8120, 8212, and 8129) had integrated the pK4 plasmid, as shown by analysis of the cellular DNA by Southern blot hybridization. As indicated by the restriction fragment pattern, the integrated plasmid DNA was grossly intact. 1–3 integrated copies were detected depending on the analyzed clone (Fig. 1, *top*). All three clones expressed the SV-40 T antigen in 100% of cell nuclei (Fig. 1, *C and D*).

Further analysis of the transformed clones (1003-pK4) revealed that the cells had remained undifferentiated, as judged by their morphology, the persistence of the expression of specific markers of EC cells (4, 23) (Fig. 1, *A and B*), and their potential to differentiate, when submitted to a suitable environment, into derivatives of the three embryonic germ layers. Except for the integration of the plasmid and the expression of the SV-40 oncogenes, the 1003-pK4 cells could not be distinguished from the parental EC 1003. We conclude that the SV-40 T antigen can be expressed in 1003-pK4 cells as early as the EC stage, and that the presence of the T antigen neither causes recognizable phenotypic change nor prevents differentiation *in vivo* or upon *in vitro* induction.

Selection of an Osteogenic Clone among Immortalized Mesodermal Derivatives. Each of the three 1003-pK4 clones was injected into irradiated syngeneic mice. Teratocarcinomas were produced within 1–3 mo in all inoculated animals. They were composed of EC cells, and of various differentiated derivatives of the three germ layers. In cultures derived from the tumor, most cells expressed the SV-40 T antigen.

One tumor arising from 1003-pK4 clone 8129 was allowed to grow *in vitro*. Areas containing mature mesodermal derivatives closely associated with immature cells were selected with the idea that the immature cells might correspond to mesodermal progenitors. Clones harboring various phenotypes were isolated from this mesodermal population by limiting dilution in Costar multidishes (0.3 cell per well). All clones expressed the SV-40 T antigen (Fig. 2, *A and B*). Among them, myoblast-like clones giving rise at confluence to multinucleated myotubes (Fig. 2, *C and D*). Some clones were enriched in adipoblasts and adipocyte-like cells containing lipid droplets (Fig. 2 *E*). Others were fibroblast-like (Fig. 2 *F*) or exhibited a mixed phenotype (Fig. 2 *H*). Finally, some clones were composed of polygonal cells of unidentified phenotype (Fig. 2 *G*). To screen for the presence of an osteogenic precursor among the immature cells, the various subpopulations were injected into syngeneic mice. The tumors formed contained only mesodermal derivatives. The polygonal cells gave rise in all cases to tumors containing mesoblastic cells, osteoblast-like cells oriented around host capillaries, and developing bone. Transitional stages between the immature cells and mineralized bone were present in all the tumors (not shown). The cell population arising from one tumor (87102) was further cloned by limiting dilution. Clones were selected on the basis of their

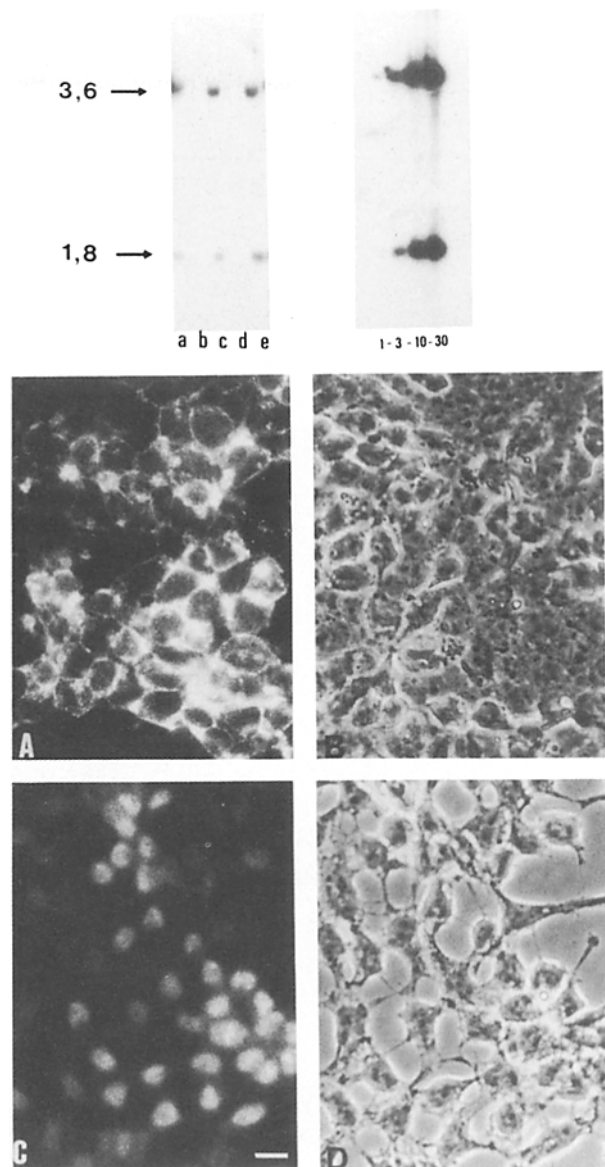


Figure 1. (*Top*) Southern hybridization analysis of genomic DNA from 1003-pK4 transfected clones. Digestion by Eco RI and Pvu II (size of the fragments: 3.6 and 1.8 kb). Lanes *a*, *c*, and *e*: transformed clones 8120, 8212 and 8129, respectively. Lanes *b* and *d*: untransformed clones 8230 and 8209. 1, 3, 10, or 30 copies of digested pK4 were used for calibration. (*Bottom*) Immunofluorescence staining of the 1003-pK4 transformed clone 8129 with *A*. ECMA seven antibodies recognizing EC cell-surface antigens. Fixation with 3% paraformaldehyde. (*C*) mAb against T antigen. Methanol fixation at -20°C . (*B and D*) Phase-contrast micrographs of the same fields. Bar, 10 μm . $\times 400$.

polygonal morphology and of their high level of bone specific heat-labile alkaline phosphatase activity (Table I). One clone, C1, was selected for further analysis.

Properties of the Osteogenic Clone C1

Characteristics of the Precursor Cell. C1 cells exhibit a polygonal morphology (Fig. 3 *A*). All the cells of the clone, ex-

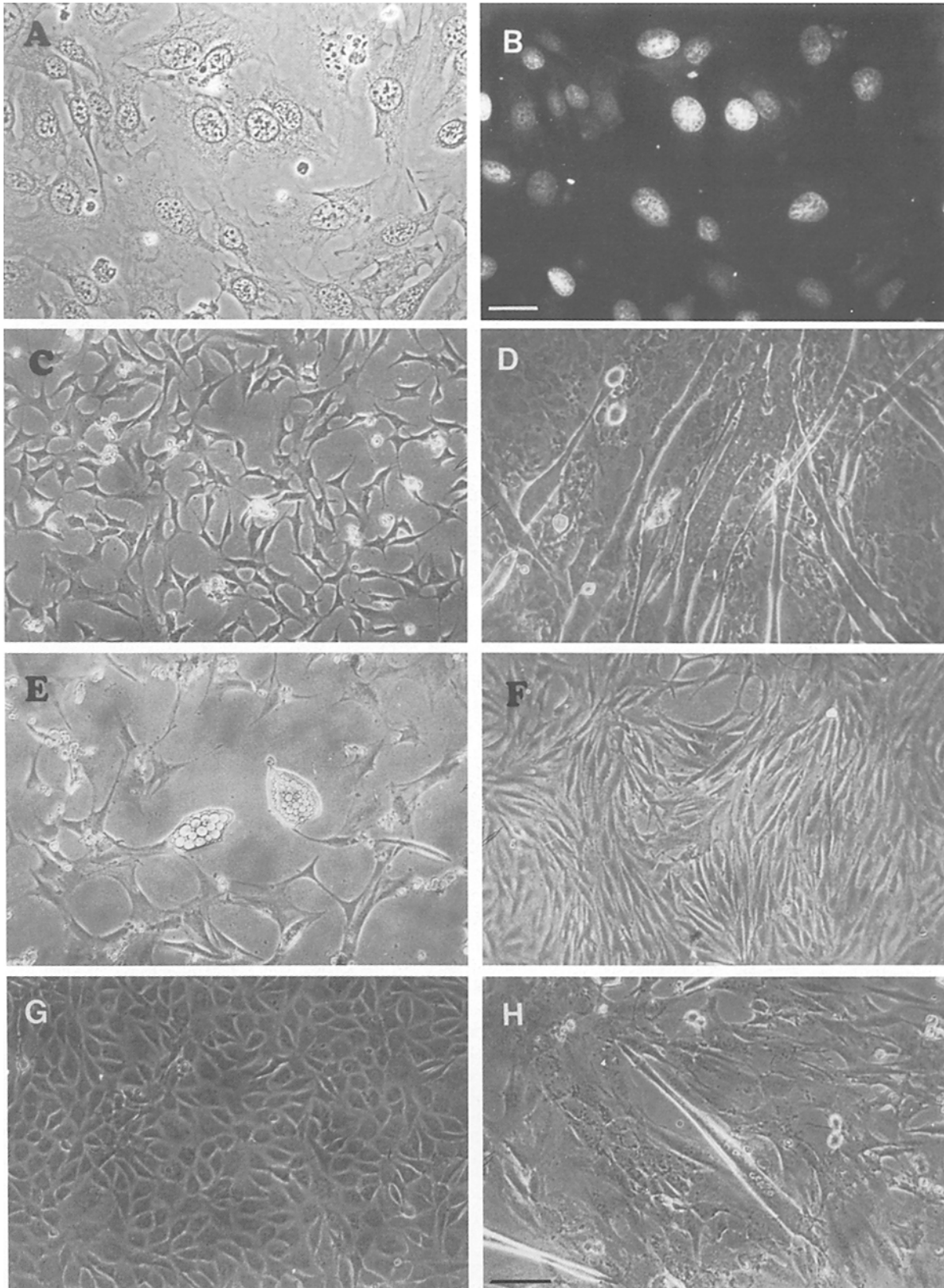


Figure 2. Phenotypes of the mesodermal clones derived from a 1003-pK4 teratocarcinoma. (A) Phase-contrast and (B) immunofluorescence labeling with mAb against T antigen of an immature, mesoblastic like clone. Bar, 10 μm $\times 800$. (C and D) Myoblast-like cells and the same clone showing multinucleated myotubes at confluence. (E) Adipocytes. (F) Fibroblasts. (G) Polygonal cells of unidentified phenotype. (H) mixed phenotype. Bar, 50 μm . $\times 200$.

Table I. Alkaline Phosphatase Activity

	Total activity	Heat-labile activity
	$\mu\text{mol PNP/mg protein per min}$	
Embryonal carcinoma 1003	0.01 \pm 0.001	0.01 \pm 0.002
Embryonal carcinoma 1003-pK4	0.01 \pm 0.002	0.01 \pm 0.001
Tumor 87102	0.21 \pm 0.02	0.20 \pm 0.02
Clone F1	0.02 \pm 0.003	0.02 \pm 0.003
Clone C1	0.23 \pm 0.05	0.27 \pm 0.03

Cells were plated in DME containing 10% FBS at 4×10^3 cells/cm². When the cells were confluent, they were first washed with DME and then cultured for a further 24 h in serum-free DME. The cells were scraped, cell lysates were collected and sonicated, and alkaline phosphatase activity was determined in cell lysates. Values are the mean \pm SEM of four independent confluent cultures.

press the SV-40 T antigen in their nucleus (Fig. 3 B). When growing exponentially, the clonal cell population has a doubling time of 30 h and maintains a stable, homogeneous phenotype for over 100 generations.

C1 cells synthesize type I collagen, a characteristic feature of osteoblastic cells (1). At confluence, collagen represents $8.2 \pm 1.4\%$ ($n = 4$) of total protein, as assessed by collagenase digestion of [³H]proline-labeled C₁ cells. SDS-PAGE analysis of the proteins digested with pepsin shows two major bands comigrating with standard $\alpha 1(1)$ and $\alpha 2(1)$ collagen chains. Collagen type III chains are not detectable (Figs. 4 and 5A).

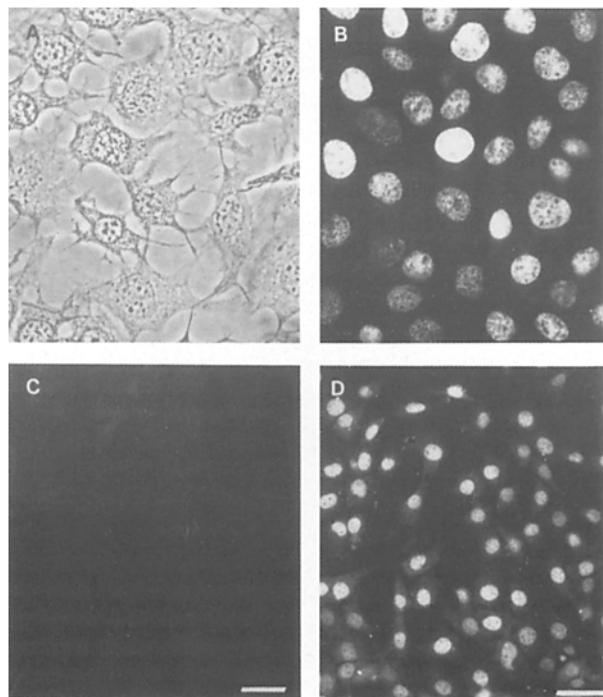


Figure 3. C1 clone. (A) Phase-contrast micrograph. (B) Immunofluorescence staining with a mAb against T antigen. (C) control without primary antibody. Methanol fixation at -20°C . Bar, 20 μm . $\times 300$. (D) Staining with anti-T antibody of a cell culture derived from C1 osteosarcoma. Bar, 50 μm . $\times 120$.

Table II. Intracellular cAMP Production by C1 Cells

	M	cAMP pmol/mg protein
Controls (PBS)		11.8 \pm 0.2*
(1-34)hPTH	10^{-10}	20.6 \pm 0.8*
	10^{-9}	49.7 \pm 9.3*
	10^{-8}	89.1 \pm 9.6*
	10^{-7}	102.7 \pm 6.2*
Controls (PBS)		7.0 \pm 1.5
CT	10^{-8}	7.1 \pm 0.8
	10^{-6}	4.4 \pm 0.8
Controls (ethanol)		2.7 \pm 0.2
PGE ₂	10^{-8}	3.6 \pm 0.4
	10^{-7}	4.3 \pm 0.25*
	10^{-6}	7.2 \pm 0.6*

C1 cells were cultured in DME with 10% FBS. At confluence cells were washed, cultured in DME and 0.1 mM isobutylmethylxanthine (IBMX) for 1 h. The cells were then washed and treated in DME with 0.1 mM IBMX for 10 min in presence of the compounds or their vehicle. The cells were scraped and cAMP concentrations were determined in cell lysates. Values are the mean \pm SEM of three to four independent confluent cultures.

* Significant difference with the appropriate control ($P < 0.05$ or better level of significance by t test).

Alkaline phosphatase activity is revealed in most of the C1 cells. The parental clones 1003 and 1003-pK4 also express alkaline phosphatase, a marker of embryonal carcinomas and of germinal cells (4). However, as illustrated in Table I, alkaline phosphatase activity levels present in extracts of C1 cells are much higher than those in 1003 and 1003-pK4, or in the fibroblastic-like clone F1. The osteosarcoma 87102 from which C1 and F1 were cloned, also shows a high level of alkaline phosphatase activity.

Moreover, most of the C1 enzymatic activity (95%) is lost upon heating to 56°C for 30 min (Table I), a characteristic of bone-specific alkaline phosphatase (12). In addition, 59.6% of the alkaline phosphatase activity ($p < 1,000$) are inhibited by physiological doses of 1,25 dihydroxyvitamin D₃ [$1,25(\text{OH})_2\text{D}$] (10^{-8} M). Furthermore, C1 cells are responsive to PTH and PGE₂ but not to CT in terms of stimulation of cAMP production, another characteristic feature of osteoblastic cells (Table II) (29, 30).

A significant amount of acid phosphatase activity is found in the cytoplasm of the cells (not shown). Such an activity was recently demonstrated in vivo in rat osteoblasts and osteocytes (5).

Differentiation In Vivo. When injected subcutaneously into irradiated syngeneic mice, the SV-40-transformed C1 cells develop tumors, found to be typical osteosarcomas. The tumors maintain their histological features after serial passages in vitro and in vivo. They form a dense trabecular bone network with extensive vascularization. Several trabeculae of calcified bone matrix are regularly arranged within the osteosarcoma. Numerous bone forming cells are dispersed along the bone matrix, some of which are embedded like osteocytes (Fig. 6). Bone resorption by osteoclast-like cells appears in some areas, but host marrow cells are not apparent.

C1 cells are therefore able to form and mineralize in vivo a calcified matrix similar to immature spongiosa. The osteosarcoma cells must moreover originate from the in vivo

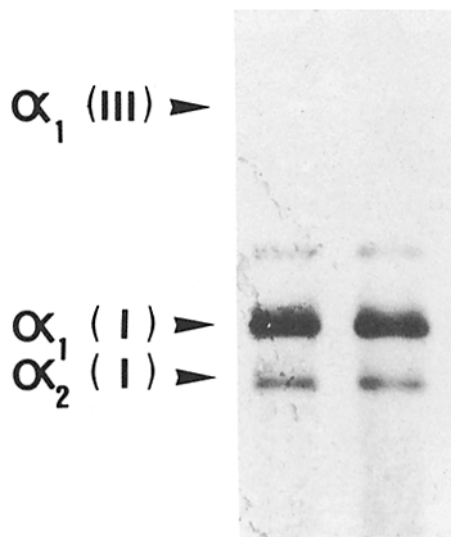


Figure 4. Collagen synthesis in confluent C1 cultures. Day 5 cultures were labeled with [³H]proline for 24 h. Cell extracts and medium were digested with pepsin, dialyzed and subjected to SDS-PAGE, with (*left*) or without (*right*) delayed reduction by β -mercaptoethanol. The mobility of the references, type I collagen α_1 (I) and α_2 (I) and of type III collagen α_1 (III) are indicated.

differentiation of C1 since they express the SV-40 T antigen in the tumor.

Differentiation and Maturation In Vitro. In vitro differentiation of cells of the immature clone C1 into cells able to mineralize was found to require several conditions: (a) the addition of ascorbic acid, which enabled C1 cells to produce an abundant extracellular matrix. Immunocytochemical (Fig. 5 A) and biochemical analysis (Fig. 4) revealed that the matrix contained type I collagen, whereas type III collagen could not be detected. (b) a clustering of the cells (see Material and Methods), which was a prerequisite for mineralization (1, 3, 8, 35), and led by day 10 to the formation of numerous large nodules composed of cells staining intensively for alkaline phosphatase activity (Fig. 5, B and C) and surrounded by a dense fibrous material. (c) In the absence of β -glycerophosphate, no calcification of the nodular structures was seen (Fig. 5 E). When β -glycerophosphate was added, the matrix reached complete mineralization as revealed by positive Von Kossa staining. The nodules were composed of mineralized bone-like matrix deposited by the C1 cells, some of which being embedded within the matrix as osteocyte-like cells (Fig. 5 F).

Electron microscopic examination showed that the untreated C1 cells were surrounded by a dense matrix of striated collagen. Upon addition of β -glycerophosphate, minerals were found to be structurally associated with the striated collagen fibrils (Fig. 7).

Prominent peaks of calcium and phosphorus were detected in the profiles of the mineralized regions (Fig. 8 A), using energy dispersive x-ray analysis. P/Ca ratios were close to the 0.6 value of hydroxy apatite (0.56 ± 0.01). Furthermore, electron diffraction patterns of the same zones demonstrated that the minerals had the characteristic crystalline structure of hydroxyapatite (Fig. 8 B).

Discussion

In a recent report (21), we have introduced a method to derive various immortalized precursors of the neuroectodermal, mesodermal, and endodermal pathways from the embryonal carcinoma F9. This method is based upon the transformation of F9 cells by a plasmid, pK4, carrying the SV-40 early genes under the control of the adenovirus Ela promoter. The transformed F9 cells differentiated upon addition of retinoic acid to the cultures (20, 21). The expression of SV-40 oncogenes in the transformed F9 cells accompanied the differentiation.

In this work, the plasmid pK4 is introduced into another EC cell line, 1003. In contrast with the case of F9 EC cells, expression of the SV-40 oncogenes in 1003 cells is realized as soon as the EC stage and does not require the induction of differentiation. Note that the oncogene expression does not induce differentiation (33, 34) and does not alter the multipotentiality of the EC cells, as indicated by the presence of the expected differentiated derivatives of the three germ layers within tumors from mice inoculated with the transformed 1003 clones. In particular, among the derivatives, committed precursors of the mesodermal lineage were cloned.

This study both generalizes and extends our earlier observations. The recombinant plasmid pK4 is clearly of a potential interest for delineating molecular mechanisms leading to the expression of the SV-40 T antigen in embryonal carcinoma cells through comparative studies of 1003 and F9 cells (24, 26, 42). pK4 also appears to be a valuable tool for establishing stable cell lines corresponding to progenitor cells. It promotes immortalization at an early stage, while still allowing the expression of differentiation programs.

This study is devoted to the characterization of an osteogenic clone, C1. C1 isolation required several steps: transfection of an embryonal carcinoma cell line known for its high efficiency of mesodermal differentiation, selection of a tumor giving rise to many mesodermal derivatives, cloning from this tumor in search of the presence of osteogenic clones among mesodermal derivatives, and finally selection of the clones for their osteogenic potential in vivo and their high activity of bone-specific alkaline phosphatase in vitro (18, 27).

C1 cells are clearly committed towards the osteogenic pathway. (a) After injection into syngeneic mice, they produce typical osteosarcomas which indeed originate from the de novo differentiation of C1, as shown by the expression of the SV-40 T antigen in the tumors. (b) In vitro, C1 cells express osteoblast phenotypic markers, including synthesis of type I collagen (1), expression of the bone-specific alkaline phosphatase isoenzyme (12) and osteogenic potential in vivo and in vitro. (c) In addition, C1 cells respond to normal bone maturation effectors, such as PTH, prostaglandins, and $1,25(\text{OH})_2\text{D}_3$ but do not respond to calcitonin. (d) Upon differentiation in nodular structures induced by ascorbic acid and β -glycerophosphate, a collagenous matrix is deposited and mineralizes forming bone-like structures, with minerals maturing into hydroxyapatite crystals.

Thus, C1 cells appear to be a reliable and convenient system to study osteogenesis in vitro as well as in vivo. Indeed, previously described rat osteosarcoma cell lines did not produce a mineralized bone matrix unless they were cultured in agar (37), or were in close contact with extracellular stimulatory factors diffusing through diffusion chambers (43). In

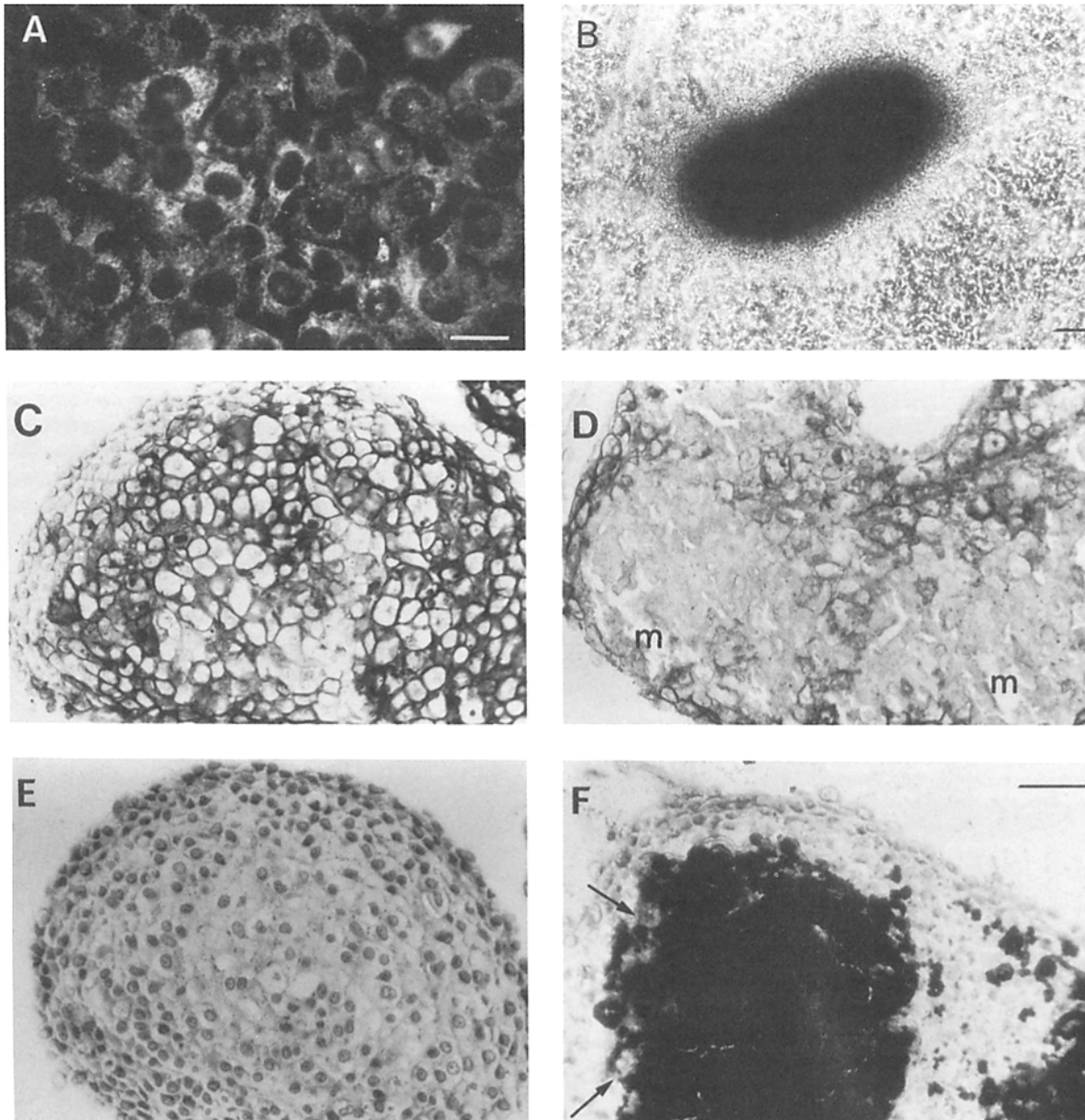


Figure 5. In vitro differentiation of C1 cells. (A) Immunofluorescence with anticollagen I antibodies showing intracellular staining of exponentially growing cells. Fixation with 3% pFA, then with methanol. Bar, 10 μm . $\times 800$. (B) Calcified nodule grown in the presence of ascorbic acid and β -glycerophosphate. Bar, 50 μm . $\times 100$. (C–F) Transverse sections (5 μm thick) of undecalcified nodules. Culture in suspension for 21 d in the absence (C and E) or in the presence (D and F) of β -glycerophosphate and ascorbic acid. (C) Intense membrane-bound alkaline phosphatase activity without mineralization. (D) Toluidine blue-stained section showing a large mineralized matrix (m) surrounded by alkaline-phosphatase-positive cells. (E) Von Kossa staining showing the absence of mineralization. (F) calcified matrix and osteocyte-like cells (arrows) embedded within the mineralized matrix. Bar, 50 μm . $\times 200$.

mice, bone formation arising after injection of a teratocarcinoma-derived untransformed cell line has been described (36). However, the resulting osteochondrosarcoma tumors were not formally related to the in vivo differentiation of the injected cells and no clonal cell line expressing in vitro an osteogenic phenotype could be obtained. It is noteworthy to mention also the work of Silbermann et al. (44) who used viruses containing an active *v-fos* oncogene to transform in vitro embryonic murine condyles. After injection in mice, highly abnormal bone-like metastatic tumors were obtained.

However, these metastatic tumors contrast with our C1 cell system, because the bone obtained upon C1 differentiation has histological features typical of normal immature bone, and since the C1-derived tumors have no metastatic properties despite the expression of the early SV-40 oncogenes. Finally, a recombinant retrovirus containing the SV-40 T antigen was recently used to immortalize rat calvaria cells. The immortalized cell lines obtained were shown to retain osteoblastic properties (15). As compared with the Calvaria cell lines, C1 cells have two additional features. Firstly, the

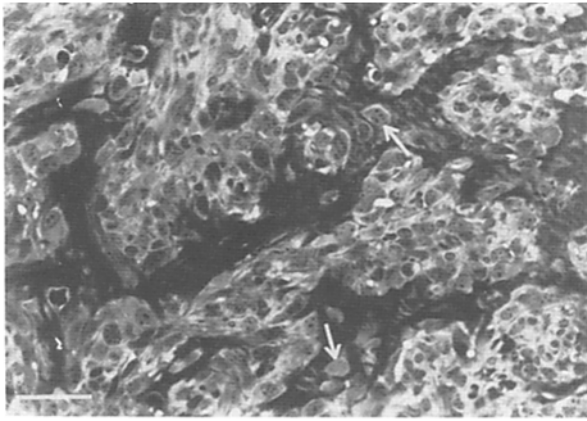


Figure 6. Section of an osteosarcoma derived from C1 cells: dense trabecular bone network with extensive vacularization. Osteocytes embedded in the bone matrix (arrows). Bar, 50 μm . $\times 200$.

cells are osteogenic in vitro, allowing the study of gene regulation during matrix mineralization. Secondly, it should be recalled that the C1 clone derives from an island of cells resembling the somitic mesoderm of the early embryo. This island was composed of mesoblastic cells able to differentiate into either myogenic, fibroadipogenic, or osteogenic derivatives. The study of various clones representative of those mesoblastic cells is now under way. Hopefully, some of these clones may correspond to immortalized stages of bone commitment earlier than the C1 stage. This idea is supported by

a recent report (13) showing that progenitor cells in a bone-derived clonal cell population from rat can differentiate into bone as well as into muscle, fat, or cartilage. Therefore, our immortalized teratocarcinoma-derived system appears to be of interest to study also the regulation of mesenchymal stem cell differentiation.

In conclusion, the teratocarcinoma-derived clone C1 offers the advantages associated with both osteosarcoma-derived cell lines having the potential for proliferation, and osteoblastic-like clones derived from normal bone, having the ability to differentiate in vitro into osteogenic cells. The potential of the C1 cells for proliferation and their stable phenotype will give the possibility to obtain large amounts of homogeneous material corresponding either to exponentially growing precursor cells or to cells induced to differentiate. Differential screening with other mesoblastic clones and with the initial embryonal carcinoma may permit the identification of specific molecular markers for the osteogenic pathway (38), the investigation of gene regulation during osteogenesis, and the study of the ontogenesis of osteoblasts.

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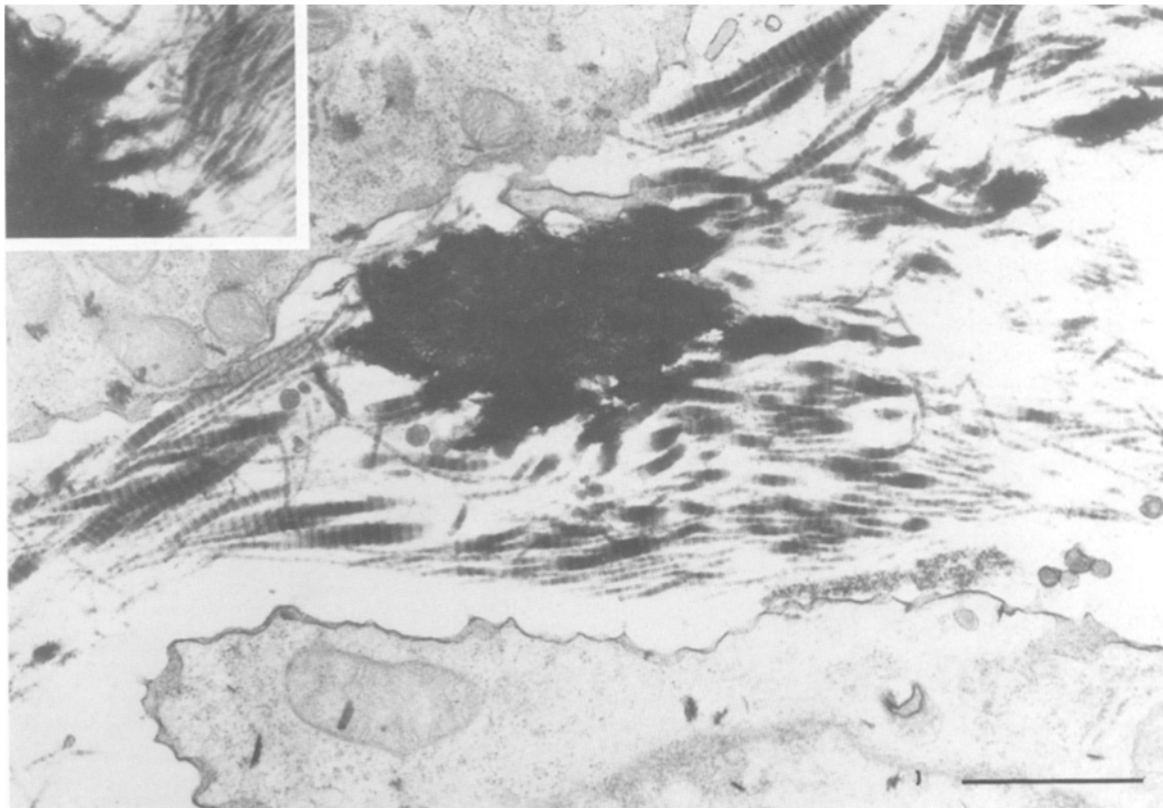


Figure 7. Electron micrograph of the mineralized matrix: C1 osteogenic cells are surrounded by banded collagen fibers. The mineralized collagen form a dense calcified matrix. Bar, 1 μm . $\times 24,000$. The deposition of minerals is closely associated with collagen fibers (inset, $\times 22,600$).

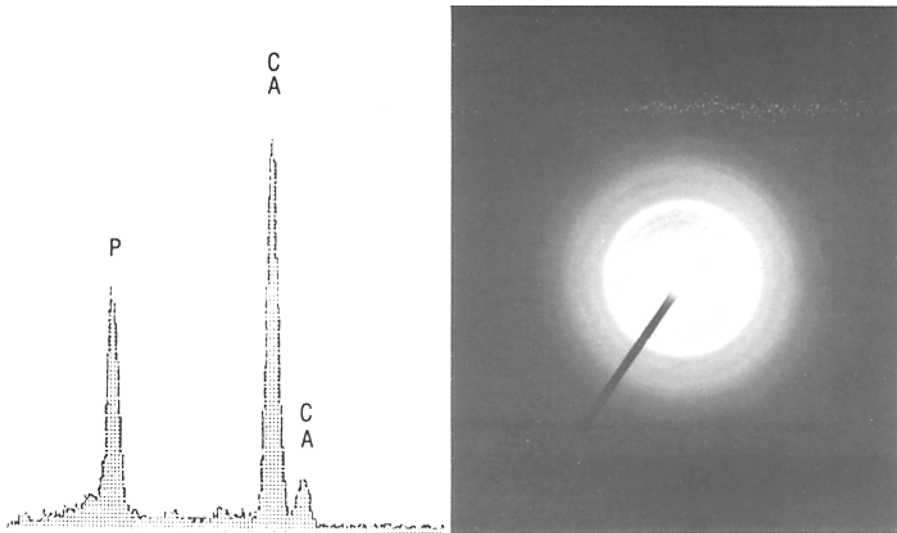


Figure 8. (A) Energy-dispersive x-ray spectrum of a mineralized area of a nodule, showing major peaks for calcium (Ca) and phosphorus (P). (B) Electron diffraction pattern of the mineral shown in Fig. 7. The mineral shows a crystalline structure characteristic of hydroxyapatite.

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