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Molecular Basis of Human Cancer

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Edited by

Claudio Nicolini
University of Genoa
Genoa, Italy

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PREFACE

During May 21-June 1 1990, the eleventh course of the International School of Pure and Applied Biostructure, a NATO Advanced Study Institute, was held at the Ettore Majorana Center for Scientific Culture in Erice, Italy, co-sponsored by the Italian Ministry of Universities and of Scientific and Technological Research, the North Atlantic Treaty Organization, the Italian National Research Council, the Sicilian Regional Government and Technobiochip. The subject of the course was "Molecular Basis of Human Cancer" with participants selected worldwide from 15 different countries.

The purpose of the course was to address, in a tutorial and structural fashion, the molecular basis of human cancer, including the mechanism of signal transduction in mammalian cells, the genetic mechanism of malignant transformation in man, growth factors, hormone receptors, cell membrane and cytoskeleton, and DNA high order structure. The course had this as its major objective and the resulting book reflects it. The participants were exposed to a critical evaluation of current knowledge about cancer and to some of the key problems that remain as stumbling blocks to our eventual understanding of this important biological and medical problem. Through the media of formal and informal lectures, workshops, symposia and informal discussions, a select group of interested young and senior scientists were acquainted with many of the aspects of human cancer.

This book is the result of this Advanced Study Institute and is the twelfth of a series, which began with "Chromatin Structure and Function" (1979, Vol. A21 & B) and continued with "Cell Growth" (1982, Vol. A38), "Chemical Carcinogenesis" (1982, Vol. A52), "Interactions between Electromagnetic Fields and Cells" (1985, Vol. A97), "Modeling and Analysis in Biomedicine" (1984, W.S.P.), "Structure and Function of the Genetic Apparatus" (1985, Vol. A98), "NMR in the Life Sciences" (1986, Vol. A107), "Cell Biophysics" (1987), "Towards the Biochip" (1988, W.S.P.), "Protein Structure and Engineering" (1989, Vol. A183), and Structure and Dynamics of Biopolymers" (1986, Vol. E133, Martinoff), edited or occasionally coedited by myself as Director of the International School of Pure and Applied Biostructure and published mostly by Plenum within the NATO ASI Life Science Series and partly by other publishers. This book aims to present a structured and interdisciplinary view of current knowledge on the possible molecular and cellular mechanisms leading to human cancer initiation and promotion.

I wish to express my gratitude to Carlo Croce for his active coleadership in the planning and conduction of the course at Erice, to Laura Vergani for her invaluable and critical cooperation prior, during and after the Institute and publication of this volume and to Maria Raffaele for the typing and editorial assistance of these proceedings.

Claudio Nicolini

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CELL STRUCTURE AND THE REGULATION OF GENES CONTROLLING PROLIFERATION AND DIFFERENTIATION: THE NUCLEAR MATRIX AND CYTOSKELETON

Gary S. Stein, Jane B. Lian, Thomas A. Owen, Janet L. Stein, Melissa Tassinari, Andre van Wijnen, Leesa M. Barone, Victoria Shalhoub, Michael Aronow, Gerry Zambetti[+], Steven I. Dworetzky*, Shirwin Pockwinse, and Joost Holthuis@

Department of Cell Biology, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655
Present address: [+]Department of Biology, Lewis Thomas Lab, Princeton University, Princeton, NJ 08544
Present address: *CNS Biology, Bristol-Myers Squibb, 5 Research Parkway, Wallingford, CT 06492
Present address: @The Central Lab for Blood Transfusion, Amsterdam, The Netherlands

In this chapter and in the one which follows we will present concepts and experimental approaches associated with the relationship of proliferation to differentiation with emphasis on the contribution of cell structure to the regulation of cell growth and tissue specific gene expression. While these relationships are of broad biological relevance, we will focus primarily on development of the osteoblast phenotype with the understanding that analogous principles apply to the regulation of phenotype expression in general.

Initially, we will review several of the fundamental elements of the sequence of events by which cell growth contributes to the onset and expression of differentiation whereby cells undergo a developmental maturation process, progressively acquiring phenotypic properties associated with specialized cells. Then, we will examine gene regulation related to expression of the osteoblast phenotype within the context of cellular architecture: first considering the potential contribution of the nuclear matrix to modifications in transcription during osteoblast growth and differentiation; then evaluating the contribution of the cytoskeleton to the stability and translatability of mRNAs.

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THE GROWTH-DIFFERENTIATION RELATIONSHIP

General Features

Although the concept of a relationship between proliferation and differentiation has been viewed as a necessary component of the developmental process for more than a century, the experimental approaches have been largely descriptive and the results primarily correlative. However, the recent development of culture systems which support the differentiation of specialized cells has permitted the combined use of biochemical, molecular and ultrastructural approaches to address the relationships of cell growth to the expression of cell and tissue-specific phenotypic properties (Figure 1). The application of in situ methodologies is particularly important in approaching questions related to differentiation since these in vitro systems support the development of a tissue-like organization analogous to that which occurs in vivo necessitating an understanding of molecular signaling mechanisms at the single cell level. Examples of such in vitro systems include: pluripotent promyelocytic leukemia cells which develop the monocytic, macrophage, or granulocytic phenotype[1,2]; adipocytes[3,4]; myoblasts[5,6]; keratinocytes[7,8]; and osteoblasts[9-14].

There are several features of the proliferation/ differentiation relationships exhibited by these in vitro systems which bear striking analogies to tissue development in

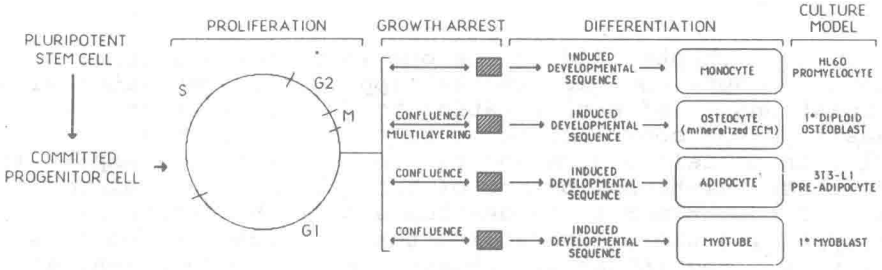

Fig. 1. Schematic representation of the events associated with progressive expression of monocyte, granulocyte, osteoblast, adipocyte and myotube phenotypes. Initially, the cells (HL-60 promyelocytic leukemia cells, primary cells of embryonic calvarial osteoblasts, 3T3-L1 pre-adipocytes or myoblasts) actively proliferate, expressing cell cycle and cell growth regulated genes, as well as genes encoding extracellular matrix proteins. Following growth arrest, a developmental sequence involving the sequential and selective expression of genes that results in the differentiated cell and tissue phenotype occurs. Completion of the proliferation period marks an important transition point where expression of tissue specific genes, often functionally related to the down-regulation of proliferation, is initiated.

vivo. In addition to validating the culture models, these relationships establish what appears to be important and possibly rate limiting steps in the differentiation process. Initially, a pluripotent stem cell and/or committed progenitor cell undergoes active proliferation, increasing the pool of precursor cells and the tissue mass to accommodate the biosynthesis of the specialized cell products required for intercellular and extracellular structural and functional properties unique to a developing tissue. While actively proliferating, these cells express genes encoding cell cycle and cell growth related proteins that support the complex and interdependent events associated with the proliferative process. Additionally the possibility should be considered, and results will be presented to support, that gene expression in proliferating cells may suppress expression of genes for later events in the differentiation process. Regulation of gene expression in proliferating cells that relate to cell cycle control is largely mediated at several post-transcriptional levels (e.g. mRNA stability and phosphorylation) providing the basis for a rapid response to accommodate cellular events which include DNA replication and mitotic division. In contrast, at the completion of proliferative activity the down-regulation of cell cycle related gene expression and the initiation of cell and tissue specific gene expression is largely controlled transcriptionally. Equally important, it should be emphasized that while the completion of proliferative activity in promyelocytes, osteoblasts, preadipocytes and myoblasts is an important point in the development of the tissue-specific phenotype, a progressive series of events occurs post-proliferatively that is necessary for the ordered development of the structural and functional characteristics of a differentiated cell -- each requiring a complex series of regulatory steps mediated at multiple levels.

THE OSTEOBLAST DEVELOPMENTAL SEQUENCE

A Temporal Pattern of Gene Expression

Normal diploid osteoblasts isolated from fetal rat calvaria (21-day rats) undergo an ordered developmental sequence during a 35 day culture period resulting in the formation of multilayered nodules of cells with a mineralized Type I collagen extracellular matrix[9-16] (Figure 2). By the combined use of molecular, biochemical, and ultrastructural analysis, the expression of cell growth and tissue-specific genes has been mapped during the progressive development of the bone cell phenotype within the context of the development of a bone tissue-like organization[12-14,16] (Figure 3). The temporal sequence of expression of genes encoding osteoblast phenotype markers in culture follows the pattern of gene expression and tissue distribution determined by in situ hybridization observed in neonatal long bones[17] and during fetal calvarial development in vivo[18] supporting the biological relevance of the osteoblast culture system (Figure 2).

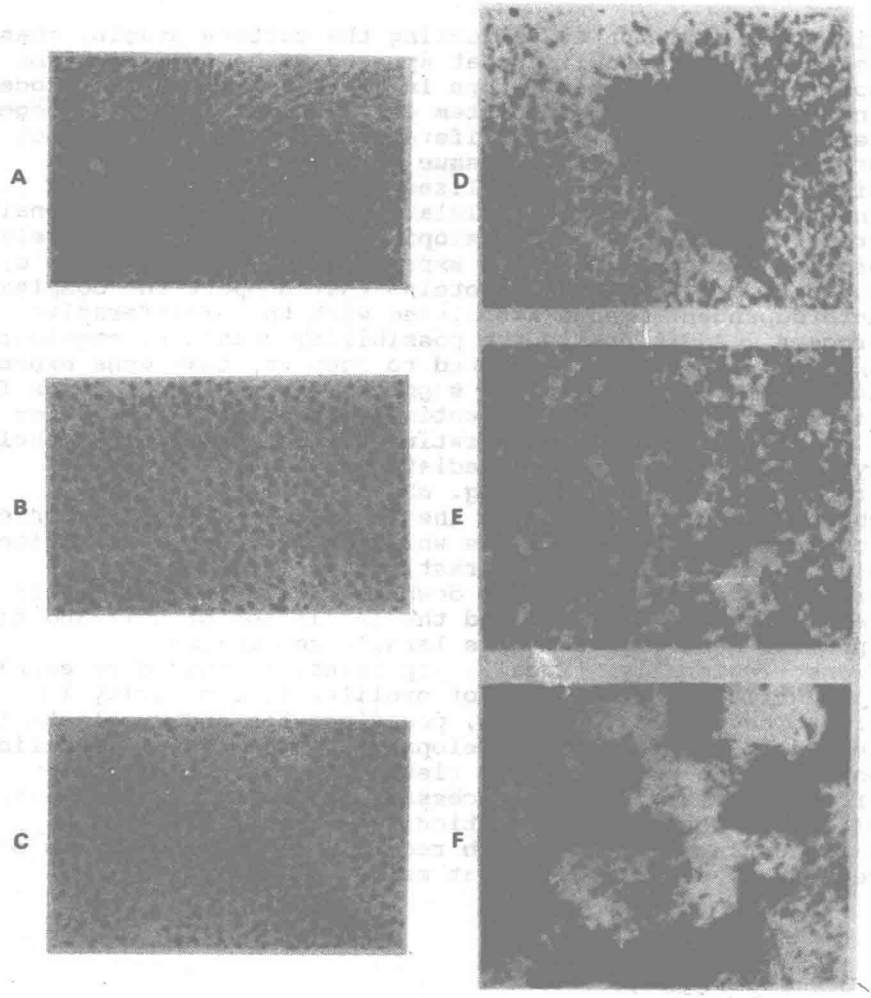

Fig. 2. Down-regulation of proliferation during the initial period of the development of the osteoblast phenotype in vitro reveals the initial cessation of proliferation in multilayered regions of the cultures. Note that on day 5 (Panel A) all cells are actively proliferating, on day 11 proliferative activity is still actively occurring throughout the cultures (Panel B), but that on day 12 there are multilayered regions of the culture where all cells have ceased proliferative activity with proliferation still ongoing in the non-multilayered regions (Panel C). The combination of alkaline phosphatase histochemistry and autoradiography following ^{3}H-thymidine incorporation shows that it is in multilayered regions of the culture that proliferation in first down-regulated and that it is these regions that first become intensely alkaline phosphatase positive (day 12, Panel D). The entire culture becomes alkaline phosphatase positive by day 16 (Panel E). The ordered deposition of mineral in nodules as extracellular matrix develops within the multilayered regions of the cells in the cultures is evident on day 35 (Panel F).

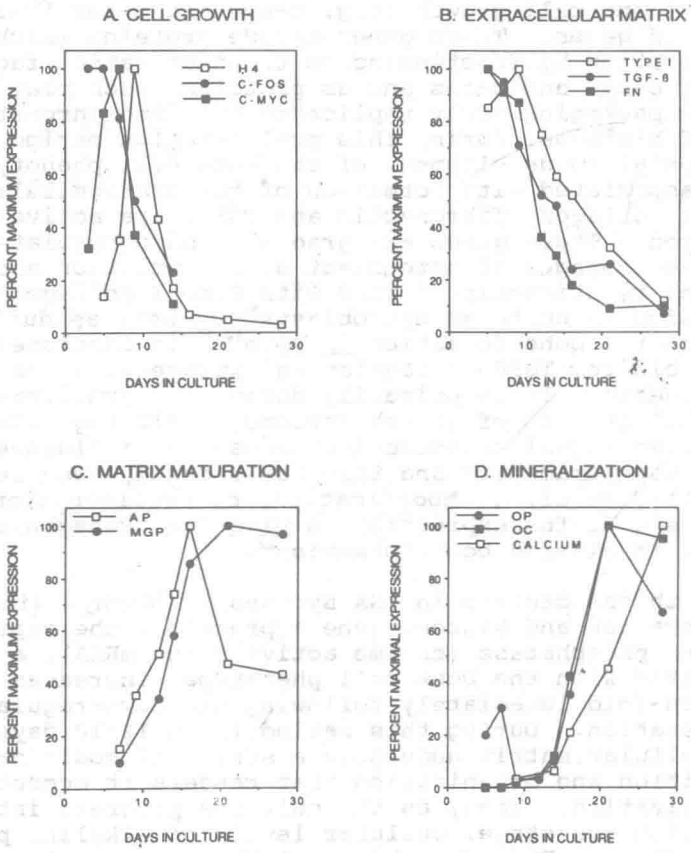

Fig. 3. Temporal expression of cell growth, extracellular matrix, and osteoblast phenotype related genes during the development of the osteoblast phenotype in vitro. Isolated primary cells were cultured after confluence in BGJb medium supplemented with 10% FCS, 50 µg/ml ascorbic acid and 10mM β-glycerol phosphate. Cellular RNA was isolated at the times indicated (3,5,7,10,12,14,16,20,28, and 35 days) during the differentiation time course and assayed for the steady state levels of various transcripts by Northern blot analysis. The resulting blots were quantitated by scanning densitometry and the results plotted relative to the maximal expression of each transcript. (A) Cell growth related genes shown are H4 histone (reflects DNA synthesis), c-myc and c-fos. (B) Extracellular matrix associated genes represented are Type I collagen, fibronectin (FN) and transforming growth factor-β (TFG-β). (C) Genes associated with extracellular matrix maturation shown are alkaline phosphatase (AP) and matrix Gla protein (MGP). (D) Genes induced with extracellular matrix mineralization represented are osteopontin (OP) osteocalcin (OC) and calcium accumulation. Note the induction of alkaline phosphatase at the end of proliferation and the induction of osteocalcin and osteopontin with the onset of calcium deposition (day 12).

Initially, during the first 10-12 days following isolation of osteoblasts from calvaria there is a period of active proliferation with expression of cell cycle (e.g. histone) and cell growth (e.g. c-myc and c-fos AP-1 activity) regulated genes. These genes encode proteins which support proliferation by functioning as transactivation factors in the case of c-myc and c-fos and as proteins which play the primary role in packaging newly replicated DNA into chromatin in the case of histones. During this proliferation period, and fundamental to development of the bone cell phenotype, several genes associated with formation of the extracellular matrix (Type I collagen, fibronectin and TGFβ) are actively expressed. These genes are gradually down-regulated during subsequent stages of osteoblast differentiation and the parallel relationship of TGFβ with Type I collagen gene expression in cultured osteoblasts[14] as well as during endochondral bone formation in vivo[19,20] is consistent with a major role for TGFβ in regulating extracellular matrix biosynthesis. It is primarily during the proliferation period that the activity of growth factors, their regulators and the associated signal transduction mechanisms influence the osteoblast parameters and this has recently been reviewed by Centrella, et al.[21]. Modifications of proliferation related genes, e.g. c-fos expression in vivo (in transgenic animals) results in altered bone formation[22].

With the decline in DNA synthesis (^{3}H-thymidine incorporation and histone gene expression) the expression of alkaline phosphatase (enzyme activity and mRNA), a protein associated with the bone cell phenotype, increases greater than ten-fold immediately following the down-regulation of proliferation. During this period (from 12-18 days) the extracellular matrix undergoes a series of modifications in composition and organization that renders it competent for mineralization. Then, as the cultures progress into the mineralization stage, cellular levels of alkaline phosphatase mRNA decline. Two other bone related genes, osteopontin[23-25] and osteocalcin[26,27], exhibit a different pattern of expression. Osteocalcin is not detectable prior to day 12 in culture and does not reach a significant level of expression until 16-20 days after isolation when expression increases coordinately with total mineral accumulation. Osteopontin similarly reaches peak levels of expression during the mineralization period (days 16-20). It is also expressed during the period of active proliferation at 25% of maximal levels. This is not an unexpected result for two reasons. First, osteopontin expression during the proliferative period is consistent with the increased level of expression during the pre-replicative phase of the cell cycle following serum stimulation of quiescent fibroblasts and following oncogene transformation or of phorbol ester treatment of fibroblasts[28]. Here one can speculate that the proliferation and tumorgenic-related function of osteopontin may be related to control of relationships between cells and extracellular matrices[28]. Second, the induced expression of osteopontin coincident with mineralization may be related to physical properties of the protein. Osteopontin is a 60 kd acidic glycoprotein[24] containing o-phosphoserine, thereby possessing several putative calcium binding sites which are known to be important

for cell proliferation and also for mineralization of the extracellular matrix in bone[29]. It therefore appears that expression of the osteopontin gene early and late in the osteoblast developmental sequence may be mediated by alternative regulatory mechanisms. Not to be overlooked is the possibility that the lower levels of osteopontin mRNA observed during the proliferation period may in part reflect mRNA transcribed *in vivo* in osteoblasts undergoing matrix mineralization prior to the isolation from fetal calvaria. The general structural and biological properties of non-collagenous proteins which are associated with the bone cell extracellular matrix has recently been reviewed by Heinegard[25].

Yet another category of genes expressed during the osteoblast developmental sequence is represented by matrix Gla protein. The matrix Gla protein gene is vitamin D responsive and the encoded 10 kd polypeptide which is associated with the osteoblast extracellular matrix contains five γ - carboxyglutamic acid residues added posttranslationally by a vitamin K dependent microsomal carboxylase. In contrast to many of the other genes expressed during specific periods of the osteoblast developmental sequence, matrix Gla protein is initially expressed in proliferating osteoblasts and expression continues during extracellular matrix maturation and mineralization. Interestingly, matrix Gla protein is not found exclusively in osteoblasts, but is also abundant in chrondrocytes and in several nonskeletal tissues with extensive extracellular matrices (e.g. lung and kidney).

The observed temporal expression of osteoblast phenotype properties during the developmental sequence is a reflection of functional activities necessary for the progressive formation of bone tissue. The expression of alkaline phosphatase mRNA and enzyme activity prior to the initiation of osteoblast mineralization suggests that alkaline phosphatase may be involved in preparation of the extracellular matrix for the ordered deposition of mineral and that the co-expression of other genes such as osteocalcin and osteopontin may support the onset and progression of extracellular matrix mineralization. Alternatively, the induction of these mineralization-associated genes may reflect an acquisition of osteoblast properties necessary for signaling bone turnover *in vivo*[30]. Taken together, the patterns of expression of these genes and the synthesis of the encoded proteins, determined biochemically and by histochemical staining, demonstrate that a temporal sequence of gene expression exists during the culture period associated with development of the extracellular matrix and reflects maturation of the osteoblast phenotype *in vitro*.

The biological relevance of the temporal expression of osteoblast parameters *in vitro* to bone formation *in vivo* is demonstrated by the fidelity of the tissue-like organization developed at the completion of the *in vitro* osteoblast developmental sequence. This is reflected by intense von Kossa silver staining of the mineralized nodules indicating hydroxyapatite deposition (Figure 2F). The bone-tissue-like organization in these cultures is further supported by

comparison of the ultrastructure of the mineralized regions of the culture. Sections through an intact 21 day fetal rat calvarium exhibits a similar ordered deposition of crystals within and between the orthogonally organized bundles of collagen fibrils. No evidence for cell necrosis or intracellular calcification is indicated in the cultures, particularly where mineralized matrix has enveloped the osteoblasts. In heavily mineralized mature cultures (after day 35), a pattern of gene expression is found analogous to the mature osteocyte in osseous tissue. In vitro alkaline phosphatase and collagen mRNA levels are almost nondetectable and osteopontin and osteocalcin levels have declined; in vivo alkaline phosphatase histochemistry indicates activity less than that found at the mineralizing front and active collagen biosynthesis is not associated with osteocytes.

Evidence for a Functional Relationship of Cell Growth to Expression of the Osteoblast Phenotype

By combining ^{3}H-thymidine labeling and in situ autoradiography with alkaline phosphatase histochemistry, it has been possible to directly establish the relationship between proliferation and initiation of tissue-specific gene expression at the single cell level during the osteoblast developmental sequence[14]. As seen in Figure 2, it is apparent that proliferation initially ceases in the discrete multilayered foci which form throughout the osteoblast cultures (Figure 2C) and it is these cells in the multilayered nodules which first express alkaline phosphatase (Figure 2D). This is in contrast to proliferating cells in the internodular regions of the cultures where alkaline phosphatase activity is not observed until several days later when the entire cultures consist of multilayered non-proliferating cells (Figure 2E).

These results confirm on an individual cell basis that a temporal sequence of gene expression occurs and that at least some events (proliferation and alkaline phosphatase expression) appear to be sequential, mutually exclusive events in the same cell, i.e., proliferation must be down-regulated prior to the expression of alkaline phosphatase. This transition from a proliferating cell to one which can express an early marker of the osteoblast phenotype (alkaline phosphatase) represents the first restriction point where cessation of proliferation appears to be required for initiation of tissue-specific gene expression associated with the distinctive characteristic features of bone -- formation of the mineralized extracellular matrix.

Another direct demonstration that the down-regulation of proliferation induces the expression of some genes which are normally expressed later in the osteoblast developmental sequence is derived from experiments which establish that inhibition of DNA synthesis in actively proliferating osteoblasts results in a rapid and selective down-regulation of cell growth genes[14] (Figure 4). This is paralleled by a four fold increase in alkaline phosphatase mRNA levels, indicating that the premature down-regulation of proliferation induces the expression of an early marker for the extracellular matrix maturation period of the osteoblast

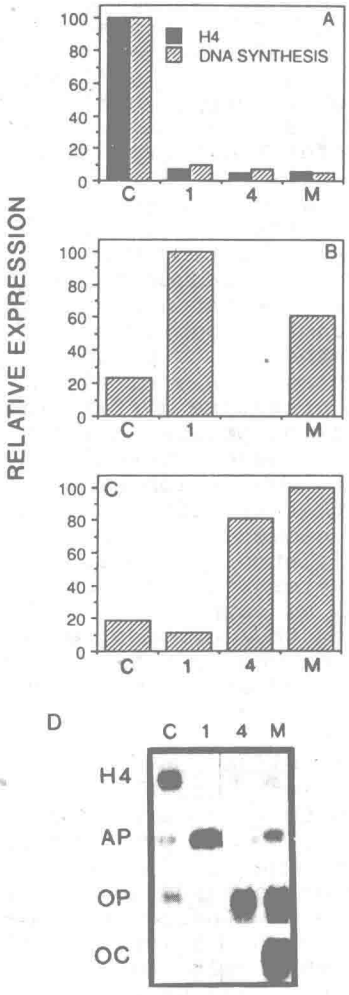

Fig. 4. Coupling of alkaline phosphatase and osteopontin but not osteocalcin expression to the down-regulation of proliferation. Proliferation was inhibited in actively growing osteoblasts (day 5) by addition of 5 mM hydroxyurea (HU). Following HU addition, cells were harvested at 1 and 4 hr., examined for DNA synthesis (A), and cellular RNA prepared and analyzed for H4 histone (A), alkaline phosphatase (B) and osteopontin (C). The Northern blots of these transcripts and osteocalcin is also shown (D). Note that osteocalcin, which is not present in 5 day cultures (C), is also not induced following inhibition of proliferation by HU. For comparison, the relative expression of these genes in non-HU-treated control (C) and in mineralized (M) cultures (30 days after plating) is also shown.

developmental sequence. Increased expression of alkaline phosphatase with decreased proliferative activity has similarly been observed in ROS 17/2.8 cells[31]. With inhibition of DNA synthesis, levels of osteopontin mRNA also increase to levels which approximate those present during the extracellular matrix mineralization period of cultured osteoblasts. These data suggest a direct functional coupling of the down-regulation of proliferation at the first transition point early during the osteoblast developmental sequence with the preferential expression of genes which are normally induced in cells with a mature extracellular matrix. However, osteocalcin which is expressed in osteoblasts late during the period of extracellular matrix mineralization, is not induced by simply inhibiting proliferative activity[14]. The absence of osteocalcin induction is consistent with the concept that there is at least a second set of genes whose expression is not directly coupled to the down-regulation of proliferation, but rather to development of the more differentiated osteoblasts in a mineralized matrix. These experiments in which premature differentiation has been promoted by inhibition of proliferation reveal a second transition point in the developmental sequence of osteoblast differentiation, since inhibition of proliferation supports expression of genes that are expressed only during progression of the developmental sequence up to the stage where mineralization is initiated. Mineral deposition may be required to signal expression of a subset of osteoblast phenotype genes, such as osteocalcin. Additionally, other genes expressed during the period of extracellular matrix maturation which may be required to render the matrix competent for mineralization may also not be induced by inhibition of proliferation.

The biological significance of the two transition points in the osteoblast developmental sequence is further suggested by the relationship between mineralization and the sequential expression of genes during the progressive development of the osteoblast phenotype. When cells are maintained under conditions which support extracellular matrix mineralization, osteocalcin mRNA and biosynthesis increase steadily beginning at day 15 in parallel with calcium accumulation in the cell layer. In contrast, when cultures are maintained under non-mineralizing conditions, calcium does not begin to accumulate in the cell layer and osteocalcin gene expression does not occur until approximately day 25; however, the presence or absence of mineralization has no effect on the expression of genes occurring during the proliferative period, on passage through the first transition point or the onset of alkaline phosphatase expression. These experiments provide additional evidence to support the existence of the second transition point, since the cells can progress through the proliferation and extracellular matrix maturation sequence to the onset of mineralization, but cannot initiate expression of genes related to the mineralization stage unless mineral accumulation occurs. Genes such as osteocalcin are not only temporally expressed late in the osteoblast developmental sequence, but are also "coupled" to deposition of hydroxyapatite.

A Model for the Relationship of Cell Growth to the Onset and Progression of Osteoblast Differentiation

The results we have presented based on determinations of molecular, biochemical and histochemical parameters are consistent with a reciprocal and functional relationship between proliferation and a sequential development of the osteoblast phenotype which is schematically illustrated in Figure 5. The progressive and interdependent series of biochemical events that characterizes the osteoblast developmental sequence reflects the selective expression, initially of genes encoding cell growth and extracellular matrix proteins and subsequently a series of tissue-specific genes[14]. Such modifications in the temporal pattern of gene expression, reflected by both the activation and repression as well as by the extent to which specific genes are expressed, are the basis for proposing a developmental sequence with three distinct periods: proliferation, extracellular matrix maturation and mineralization. This pattern of gene expression suggests two principal transition points in the osteoblast developmental sequence where important regulatory signals may be required for the progressive expression of the bone cell phenotype to proceed: the first when proliferation is down-regulated and gene expression associated with extracellular matrix maturation is initiated, and the second transition at the onset of mineralization.

Although unquestionably a simplification of an extremely complex series of biological interactions, the temporal pattern of expression suggests a working model for the relationship between growth and differentiation whereby genes involved in the production and deposition of the extracellular matrix must be expressed during the proliferative period for the onset and progression of differentiation to occur. One can postulate that proliferation is functionally related to the synthesis of a bone specific extracellular matrix and that the maturation and organization of the extracellular matrix contributes to the shutdown of proliferation which then promotes expression of genes that render the matrix competent for mineralization, a final process that is essential for complete expression of the mature osteoblast phenotype. The onset of extracellular matrix mineralization and/or events early during the mineralization period may be responsible for the down-regulation of genes expressed during extracellular matrix maturation and organization. Clearly, in this model the development of an extracellular matrix is integrally related to the differentiation stages and numerous studies have shown enhancement of osteoblast phenotype properties in the presence of ascorbic acid (summarized in references[13,14]) and under a variety of conditions which promote extracellular matrix biosynthesis and organization (see the next chapter). This working model provides a basis for addressing whether particular stages of osteoblast differentiation exhibits selective responsiveness to actions of hormones and other physiologic factors that influence osteoblast activity and other questions related to the molecular mechanisms associated with bone formation.