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PROSPECTS

Phenotype Suppression: A Postulated Molecular Mechanism for Mediating the Relationship of Proliferation and Differentiation by Fos/Jun Interactions at AP-1 Sites in Steroid Responsive Promoter Elements of Tissue-Specific Genes

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Abstract There is a generalized reciprocal relationship between cell growth and expression of genes that occurs following completion of proliferation, which supports the progressive development of cell and tissue phenotypes. Molecular mechanisms which couple the shutdown of proliferation with initiation of tissue-specific gene transcription have been addressed experimentally in cultures of primary diploid osteoblasts that undergo a growth and differentiation developmental sequence. Evidence is presented for a model which postulates that genes transcribed post-proliferatively are suppressed during cell growth by binding of the Fos/Jun protein complex to AP-1 promoter sites associated with vitamin D responsive elements of several genes encoding osteoblast phenotype markers (Type I collagen, alkaline phosphatase, osteocalcin).

Key words: oncogenes, osteoblasts, osteocalcin, alkaline phosphatase, collagen, transcription, gene expression

A functional relationship between cell growth and the initiation, as well as progression, of events associated with differentiation has been acknowledged for more than a century as a fundamental concept of development. However, the experimental approaches on which the concept of a proliferation/differentiation relationship has been based, although compelling, are largely descriptive and the results primarily correlative. By the combined application of histochemical, biochemical, and, more recently, determinations of cellular mRNA levels and rates of transcription using cloned gene probes, an extensive series of parameters associated with the sequential expression of cell growth and tissue-specific genes during cell and tissue specialization has been established. In general, proliferation appears to accompany and potentially support expression of some phenotypic genes, e.g., those associated with the biosynthesis of the extracellular matrix, while the termination of proliferative activity parallels the initiation of expression of other genes for more advanced stages of phenotype development. These relationships have, to a large extent, been observed in vivo during development as well as during tissue repair and regeneration. Additionally, the recent development of culture systems that support the differentiation of specialized cells (e.g., pluripotent promyelocytic leukemia cells that develop the monocytic, macrophage, or granulocytic phenotype [1,2]; adipocytes [3,4]; myoblasts [5,6]; keratinocytes [7,8]; and osteoblasts [9–14]) has significantly facilitated examining regulation of the expression of tissue-specific phenotypic properties. A more direct indication of a functional relationship between the down-regulation of cell growth and initiation of gene expression associated with a more mature cell phenotype is reflected by the induction of alkaline phosphatase gene expression following inhibition of proliferation in osteoblasts [14] and upregulation of muscle specific genes when growth is arrested and accompanied by myoblast fusion [5,6]. In addition to a general reciprocal relationship between cell growth and the expression of genes that occurs immediately following the completion of proliferative activity,

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the more specific possibility should also be considered that genes expressed during proliferation may result in suppression of genes at a series of stages throughout the development of the differentiated phenotype. Experimental results to support this concept will be the theme of this article.

While the sequential expression of genes during the differentiation process is mediated at the transcriptional as well as at a series of posttranscriptional levels, transcriptional control of cell growth and tissue-specific genes is an important component of developmental regulation. Despite the complex and interdependent signaling mechanisms that are operative at multiple levels, including the extracellular matrix and membrane-mediated events that through a series of second messengers may modulate mRNA stability or be transduced to the nucleus, a key consideration must be the mechanisms by which genes are selectively and sequentially rendered transcribable at specific stages during cell and tissue differentiation. Equally important are the mechanisms by which genes are suppressed prior to and following expression. Two questions that must therefore be addressed to understand transcriptional control of differentiation are, first, the identification and characterization of transcriptional regulatory elements and the promoter binding factors that interact in a sequence-specific manner to modulate specificity and level of transcription, and, second, the mechanisms by which regulatory elements are rendered competent to bind cognate transcription factors at specific times during the differentiation process.

The sequential expression of genes during progressive development of the rat osteoblast phenotype has recently been described in vivo [15–17] and in primary cultures of normal diploid osteoblasts [13,14,18, and Figure 1]. Initially, proliferating osteoblasts express cell cycle (e.g., histone) and cell growth (c-myc, c-fos, c-jun) regulated genes [14,19,20]. Also, during the proliferative period, there is expression of several genes associated with formation of the extracellular matrix (collagen, fibronectin, and TGF-β) that is essential to development of the bone cell phenotype. Immediately following the down-regulation of proliferative activity, genes such as alkaline phosphatase are expressed, during which time the osteoblast extracellular matrix undergoes a series of modifications in composition and organization that render it competent for mineralization. Then, with the onset of extracellular matrix mineralization, genes such as osteocalcin and osteopontin are expressed at elevated levels.

Several genes expressed selectively at various stages of the osteoblast developmental sequence are transcriptionally modulated by the steroid hormone vitamin D through binding of the hormone to a cytoplasmic receptor that is then translocated to the nucleus as a hormone-receptor complex where sequence-specific interactions at the vitamin D-responsive elements in the 5′ regulatory regions of vitamin D-responsive genes occur. These genes include Type I collagen, expressed during proliferation and post-proliferatively [14,21]; alkaline phosphatase [22], expressed during the periods of extracellular matrix maturation and organization; and osteocalcin, expressed during extracellular matrix mineralization [14,23]. Moreover, the biological relevance of such transcriptional modulation by vitamin D is supported by the well-documented role of vitamin D as a physiologic mediator of expression of these genes in vivo and in vitro (reviewed in [24–26]). Thus, the mechanism by which the vitamin D-responsive elements of these developmentally expressed genes exhibit occupancy by the vitamin D receptor complex is essential to our understanding of transcriptional regulation during osteoblast differentiation.

An indication of a potential molecular mechanism for mediating a relationship between proliferation and transcription of a tissue-specific gene was provided several years ago when the sequence of the bone-specific osteocalcin gene promoter was reported [23,27–30] and a series of AP-1 consensus sequences were identified in the 5′ regulatory region [23,31]. The proto-oncogene-encoded Fos and Jun proteins form a stable heterodimeric complex via a leucine zipper that interacts in a sequence-specific manner with AP-1 sites. The presence of AP-1 consensus sequences in the osteocalcin gene promoter presented the possibility that Fos and Jun proteins in proliferating osteoblasts could suppress osteocalcin gene transcription until late in the development of the bone cell phenotype, at which time extracellular matrix mineralization is initiated (Figures 1 and 2). Such a line of reasoning has recently been supported by four experimental results:

1. Identification of a vitamin D-responsive element (VDRE) in the osteocalcin gene pro-

Collagen:	CTGGGGGCAGAAGAACTTTCTGGAGGATTTGAGTGA[a] GGAGTCAGACATGGGGTGAAGGCTGTCA[b]
Alkaline Phosphatase:	GGGGGTGACTGATGGTAACCTGATTG
Osteocalcin:	CTGGGTGAATGAGGACATTACTG

Fig. 1. Vitamin D responsive element motifs. The presence of AP-1 sites within the VDRE's of the rat osteocalcin gene promoter and putative VDRE of the human alkaline phosphatase promoter [38] and an AP-1 site contiguous to, but not overlapping, the vitamin D receptor binding domain of two VDRE's [a,35; b, A. Lichtler and D. Pavlin, personal communication] in the rat Type αI collagen gene promoter.

moter (Figure 1) (nt −462 to −440 for the rat gene and nt −513 to −493 for the human gene) by deletion mutagenesis [32–34] and by direct determination of protein-DNA interactions in the 5′ regulatory sequences [31] indicated that an AP-1 consensus sequence resides within this regulatory element that mediates vitamin D enhancement of expression. Additionally, an AP-1 consensus sequence was identified in the osteocalcin box (nt −76 to −99 for the rat gene and nt −123 to −100 for the human gene) that contains a CCAAT motif as a central element and influences tissue-specific basal levels of osteocalcin gene transcription. These results are consistent with a model in which coordinate occupancy of the AP-1 sites in the VDRE and osteocalcin box in proliferating osteoblasts may suppress both basal level and vitamin D-enhanced osteocalcin gene transcription, a phenomenon described as *phenotype suppression*. A similar organization of the VDRE and osteocalcin box for the human and rat osteocalcin gene suggests that the functional properties of the elements with respect to the relationship of Jun-Fos interactions to vitamin D receptor binding are conserved.

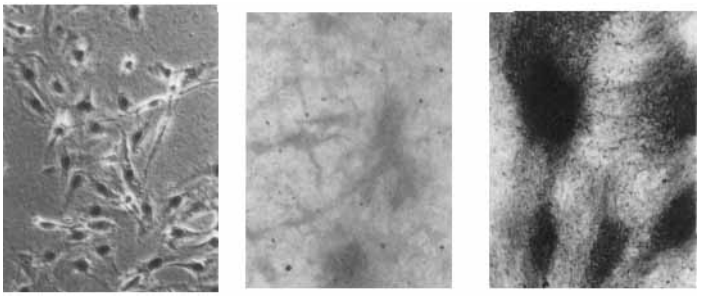
2. Sequence-specific binding of the Jun-Fos complex to the AP-1 sites within the VDRE and osteocalcin box of the osteocalcin gene promoter have been demonstrated at single nucleotide resolution [35].

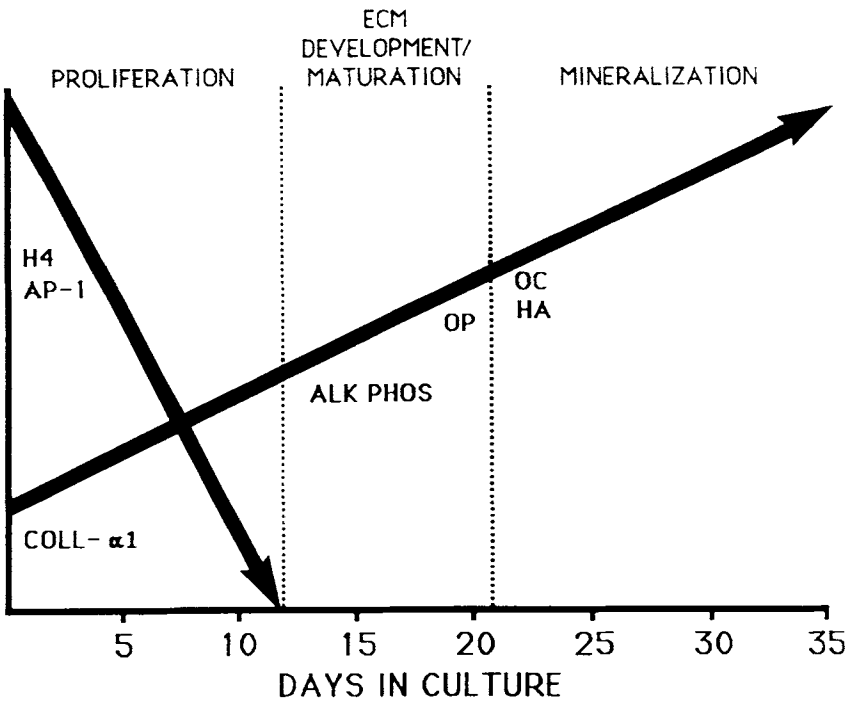
3. Expression of C-fos and C-jun have been shown to occur primarily during the proliferative period of the osteoblast developmental sequence [14,20]. Similarly, electrophoretic mobility shift analysis has indicated that AP-1 binding activity is observed primarily in proliferating osteoblasts and dramatically decreases after the down-regulation of proliferation and the initiation of extracellular matrix maturation and mineralization, at which time (coincident with) osteocalcin gene transcription is initiated [35].

4. Experiments in which transfection of C-fos and C-jun into cells expressing osteocalcin results in the down-regulation of osteocalcin gene transcription further supports a Fos-Jun-mediated suppression of the osteocalcin gene [36].

A question that must always be addressed with respect to the validity of a model proposed to explain transcriptional regulation of a gene is the extent to which the hypothesis is applicable to other genes expressed under similar or related biological circumstances. Further support for the biological relevance of postulating that Fos-Jun binding to an AP-1 site within a VDRE can suppress expression of genes normally expressed following down-regulation of proliferation comes from the organization of the alkaline phosphatase gene (Figure 1). Similar to the observed Fos-Jun interactions with the osteocalcin gene VDRE, an AP-1 motif within the VDRE of the alkaline phosphatase gene, which is also expressed following proliferation in osteoblasts undergoing differentiation, binds the Fos-Jun complex [35]. An analogous mechanism for suppression of both the alkaline phosphatase and the osteocalcin gene is particularly interesting, since while these genes are both expressed after the completion of proliferation in normal diploid osteoblasts, they are expressed sequentially: Alkaline phosphatase is expressed immediately following the down-regulation of cell growth, while osteocalcin is expressed only in the mature osteoblast undergoing the final stages of differentiation.

Yet to be addressed is the mechanism by which the osteocalcin and alkaline phosphatase genes are rendered transcribable and vitamin D responsive at specific times during expression of the osteoblast phenotype following the down-regulation of proliferation (completion of the cell growth period). However, the results are consistent with *a common mechanism for suppressing expression of osteoblast genes* transcribed post-proliferatively by Jun-Fos binding when the cells are actively proliferating and *a gene-specific mechanism for the sequential activation of these genes* during the subsequent expression of the osteoblast phenotype. Here, the possibilities include 1) release of the Fos-Jun complex from the AP-1 sites to permit sequences to be available for occupancy by the vitamin D receptor complex and/or by tissue-specific osteocalcin box




ECM
DEVELOPMENT/
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PROLIFERATION
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H4
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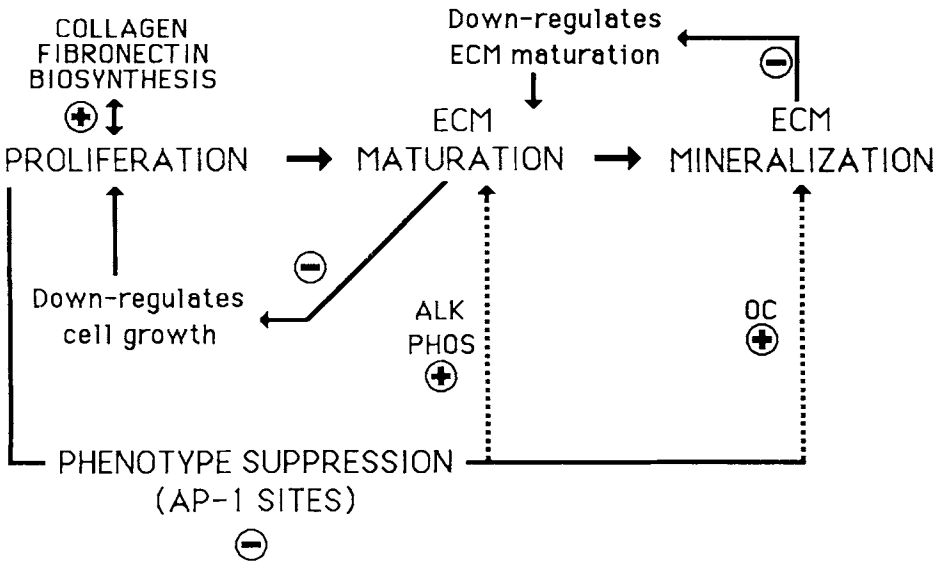
DAYS IN CULTURE


COLLAGEN
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Down-regulates
ECM maturation
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ECM
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MATURATION
MINERALIZATION
Down-regulates
cell growth
ALK
PHOS
OC
PHENOTYPE SUPPRESSION
(AP-1 SITES)

Figure 2.

transcription factors; or 2) modifications of the Fos-Jun complex that facilitates binding of activation-related factors. With respect to the latter possibility, binding of the Fos-Jun complex may pleiotropically play a dual positive and negative role in the regulation of transcription, suppressing transcription when proliferation is ongoing by directly or indirectly modulating sequence-specific interactions at the vitamin D receptor binding domain and then serving to facilitate vitamin D receptor binding post-proliferatively to facilitate the sequential upregulation of vitamin D-responsive genes.

Not to be overlooked is the well-documented vitamin D responsiveness of the Type I collagen gene that is actively expressed in proliferating osteoblasts, providing transcripts to support biosynthesis of the principal component of the bone cell extracellular matrix. Is this incompatible with the postulated involvement of Fos-Jun/AP-1 interactions in the control of vitamin D-mediated transcriptional regulation? *Not necessarily.* The sequence of the rat Type I collagen gene has recently been published by Lichtler et al. [37], and an examination of the promoter has revealed two VDRE sequences analogous to that found in the osteocalcin and alkaline phosphatase 5′ regulatory regions, but with the AP-1 consensus sequences contiguous to and not within the VDRE's (Figure 1) [35]. Sequence-specific binding of the Fos-Jun complex to the AP-1 site (Figure 1a) associated with the collagen promoter VDRE [35] indicates that subtle variations in the organization of the VDRE and AP-1 motifs in the osteocalcin and alkaline phosphatase genes compared to that in the Type I collagen gene promoter may contribute to the differential expression during the osteoblast developmental sequence.

It remains to be established whether the organization of steroid receptor binding domains and AP-1 sites within promoters of genes that are hormone responsive can provide a general mechanism for the phenotype suppression and/or activation in different periods of cell and tissue differentiation. However, support for such a model is provided by the association of AP-1 sites within consensus sequences for other steroid responsive elements including those for glucocorticoids and estrogen. Undoubtedly, the phenotype suppression model represents a simplification of an extremely complex series of protein-DNA interactions whereby multiple physiological signals are transduced to the nucleus, resulting in modifications in transcription that progressively alter the phenotypic properties of cells and lead to structural and functional events associated with differentiation. However, the strength of such a model is that it provides a basis for experimentally addressing the organization of regulatory elements for genes expressing phenotypic markers of cell differentiation within the context of responsiveness to the activity of genes associated with proliferation. Whether this is reminiscent of or directly reflecting a molecular parameter of the long-standing postulated association of proliferation and differentiation remains to be established.

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Fig. 2. Model for suppression of genes associated with the developing and mature osteoblast phenotype in actively proliferating cells by protein binding to AP-1 sites in the vitamin D responsive element (VDRE) of the alkaline phosphatase and type I osteocalcin gene promoters.

Top Panel. Light micrographs of the three principal periods of the osteoblast developmental sequence—proliferation (^{3}H thymidine autoradiography), matrix development and maturation (alkaline phosphatase histochemical stain) and mineralization (mineralized nodules, von Kossa silver stain).

Middle Panel. The relationship between proliferation and differentiation is schematically illustrated within the context of down-regulation of proliferation (H4 histone and AP-1 binding activity) and the up-regulation of genes related to maturation and mineralization (hydroxyapatite deposition) (HA = calcium + phosphate) of the osteoblast extracellular matrix (ECM). The temporal expression of genes characteristic of the osteoblast phenotype, type αI collagen (coll-αI), alkaline phosphatase (ALK PHOS), osteopontin (OP) and osteocalcin (OC) are indicated. The vertical broken lines separate the 3 periods and designate biologically significant transition points which cells can proceed up to, but not pass beyond, without specific signals.

Lower Panel. The proliferation period supports the synthesis of a Type I collagen/fibronectin ECM which continues to mature and mineralize. The model also postulates that the formation of this matrix initially down-regulates proliferation and matrix mineralization and subsequently down-regulates the expression of genes associated with ECM development and maturation period. The occupancy of the AP-1 sites in the OC box and VDRE of the osteocalcin gene and alkaline phosphatase VDRE by Fos-Jun and/or related proteins suppresses the basal and vitamin D induced expression of the alkaline phosphatase and osteocalcin genes prior to the initiation of basal expression. Fos-Jun binding to the AP-1 Site contiguous to the Type αI collagen VDRE is compatible with and may support transcription in proliferating osteoblasts.

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