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NUCLEAR STRUCTURE - SKELETAL GENE EXPRESSION INTERRELATIONSHIPS

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1. ABSTRACT

Using the osteocalcin gene as a paradigm for bone tissue-specific transcription, evidence is presented for functional linkage of nuclear architecture with developmental and steroid hormone-responsive control. The involvement of chromatin structure, nucleosome organization and the nuclear matrix is evaluated within the context of integrating regulatory signals that activate and/or suppress transcription of the osteocalcin gene. Mechanisms are evaluated which direct the bone and hematopoietic-specific acute myelogenous leukemia/core binding factor (AML/CBF) transcription factors to nuclear matrix-associated subnuclear domains that are competent to support expression.

2. INTRODUCTION: AN ASSESSMENT OF THE REQUIREMENTS FOR FUNCTIONAL INTERRELATIONSHIPS BETWEEN NUCLEAR STRUCTURE AND GENE EXPRESSION

Selective expression of genes during bone cell differentiation support proliferation and differentiation as well as maintenance of skeletal phenotypic properties. There are requirements for responsiveness to a broad range of physiological regulatory signals and the integration of activities at multiple, independent gene promoter elements.

This interfacing of regulatory cues must be sufficiently flexible to accommodate transient gene expression for developmental and homeostatic control. Sustained expression of bone tissue-specific genes is essential in specialized skeletal cells and tissues.

At one time it was anticipated that control of gene expression could be completely understood from nucleotide sequences within and flanking genes. This expectation has not been realized. The extensive database of gene sequences, promoter elements and regulatory factors developed over the past several years provides insight into parameters of transcriptional control and transcript processing. However, it is becoming increasingly evident that the linear representation of gene regulatory information is necessary but insufficient to support the plasticity of gene regulatory mechanisms that must be operative *in vivo*.

Functional interrelationships between nuclear structure and gene expression are emerging. Evidence is accruing that the regulatory information encoded in promoter sequences is rendered accessible to transcription factors by remodelling of chromatin structure and nucleosome organization (1). Nucleosomal architecture regulates competency for crosstalk between promoter domains. Modifications in chromatin architecture have been documented during development, in response to steroid hormones and within the context of cell cycle and growth control as well as differentiation (reviewed in (2,3)). Nuclear reorganization is the striking and clinically relevant hallmark of many cancer cells. In addition to providing diagnostic markers for transformation and tumor progression, these alterations in components of nuclear structure reflect abrogation of regulatory mechanisms which mediate cell growth and phenotypic control. Recently there have been significant reports of nuclear reorganization in several neurological disorders, extending the paradigm of a requirement for structural integrity of the nucleus to support fidelity of gene expression.

Historically, control of gene expression was conceptually and experimentally pursued as independent and minimally integrated questions. This independent pursuit of nuclear structure, compartmentalization and function has occurred in parallel with the appreciation that several components of nuclear architecture are associated with parameters of gene expression or control of specific classes of genes. There is longstanding acceptance that the nucleolus is the site of ribosomal gene expression. The nuclear pore is recognized as a site for facilitating the import and retention of gene regulatory factors as well as the export of gene transcripts ((4) and reviewed in (5)). SC35 domains have been extensively studied from the standpoints of RNA splicing and the dynamic recruitment of transcript processing factors (6-11). Promyelocytic leukemia (PML) bodies and coiled bodies have been associated with control of gene expression and undergo modifications in structure and potentially function in cancer cells (9,12-14). These components of nuclear architecture have been defined by immunoreactive proteins and/or ultrastructural imaging as well as by biochemical criteria. In several instances, colocalization of genes and/or gene transcripts with domains that are associated with nuclear structure has been demonstrated. A viable basis has been established for linkage between the subnuclear compartmentalization of genes, transcripts and regulatory factors with control of activity.

The rules which govern interrelationships between nuclear structure and gene expression remain to be established. However, several structural and functional components of nuclear architecture are conducive to experimentally addressing the interfacing of morphology and enzymology with gene regulatory mechanisms. Understanding of gene organization in a three-dimensional context has been significantly facilitated by a transition from the descriptive to the mechanistic pursuit of chromatin structure and nucleosome organization. For many years, studies of chromatin were dominated by high resolution ultrastructural and biophysical analyses with the objective of precisely defining structural features of the histone-DNA complexes under *in vivo* and *in vitro* conditions. But recently, pursuit of regulatory mechanisms which interrelate nuclear structure and function have been successful, (reviewed in (1)). Genetic and biochemical approaches have defined factors and sequences which mediate "heterochromatinization", accessibility of nucleosomal DNA to transcription factors and integration of activities at multiple promoter elements (reviewed in (15,16)). There have been important advances in characterizing activities involving nuclear pores. Biochemical and morphological determinants for nuclear import, export and retention have provided valuable insight into the regulated and regulatory features of this principal interface for informational exchange between the nucleus and cytoplasm (4). Similarly, there have been significant increments in our understanding of contributions by the anastomosing network of nuclear matrix fibers and filaments to control of gene expression at the transcriptional and post-transcriptional levels. Initial studies indicated that the representation of nuclear matrix proteins reflect cell and tissue phenotypic properties as well as modifications in gene expression which occur during differentiation and in tumors (17-23). Evidence directly implicates nuclear matrix-associated proteins with DNA replication and control of gene expression. The recent identification of specific regions of transcription factors which are responsible for intranuclear trafficking of regulatory proteins to the nuclear matrix-associated sites which support transcription reinforces the linkage of nuclear structure to regulation of genes (24).

This thematic issue focuses on signaling mechanisms operative in bone cells. Traditionally defined, signaling depicts the interchange of regulatory information between subcellular compartments that control gene expression. In this article we will broaden the definition of cellular signaling to encompass participation of nuclear architecture in modulating the integration of regulatory signals that control transcription. Using the osteocalcin gene as a paradigm for bone-specific transcriptional control, we will review the contributions of multiple levels of nuclear organization to regulation of expression. We will explore the concept that association of genes and cognate factors with the nuclear matrix may support the formation and/or activities of nuclear domains that facilitate skeletal transcriptional control. We will review several lines of evidence that are consistent with the hypothesis that association of AML/CBF transcription factors with the nuclear matrix may be obligatory for fidelity of gene expression and maximal transcriptional activity.

3. THE OSTEOCALCIN GENE AS A PARADIGM FOR BONE-SPECIFIC TRANSCRIPTIONAL CONTROL

3.1. Biological Parameters of Osteocalcin Gene Transcription

Signaling pathways associated with transcriptional control of the osteocalcin gene must be understood within the context of multiple biological parameters. Tissue-specific, phenotype restricted and developmental stage-specific expression is initiated at basal levels postproliferatively. Transcription is upregulated to maximal levels in response to vitamin D which functions as a physiological enhancer rather than an activation factor. Therefore, a comprehensive insight into osteocalcin gene transcription requires characterization of promoter regulatory sequences and cognate factor interactions which support the positive and negative regulatory mechanisms that determine the level at which the osteocalcin gene is expressed. In addition, it is necessary to establish the structural and functional parameters of control which integrate the activities at the various independent promoter domains and the determinants of mutual exclusive protein/DNA and protein/protein interactions which occur as a function of tissue type and developmental stage. While the complexity of osteocalcin gene regulation is rapidly increasing, a basis is emerging for support of the physiological requirements that are linked to biological control.

3.2. Functional Mapping of Osteocalcin Gene Promoter Domains

The bone-specific osteocalcin gene is organized to support responsiveness to homeostatic mediators and developmental expression in relation to bone cell

differentiation. The regulatory sequences illustrated in (figure 1) have been established in the osteocalcin (OC) gene promoter and coding region by one or more criteria that includes: (1) demonstration of an influence on transcriptional activity by deletion, substitution or site specific mutagenesis *in vitro* and *in vivo*; (2) identification and characterization of sequence specific regulatory element occupancy by cognate transcription factors *in vitro* and *in vivo*; (3) modifications in protein-DNA interactions as a function of biological activity; and (4)consequential modifications in functional activity following overexpression or suppression of factors which exhibit sequence-specific recognition for regulatory domains.

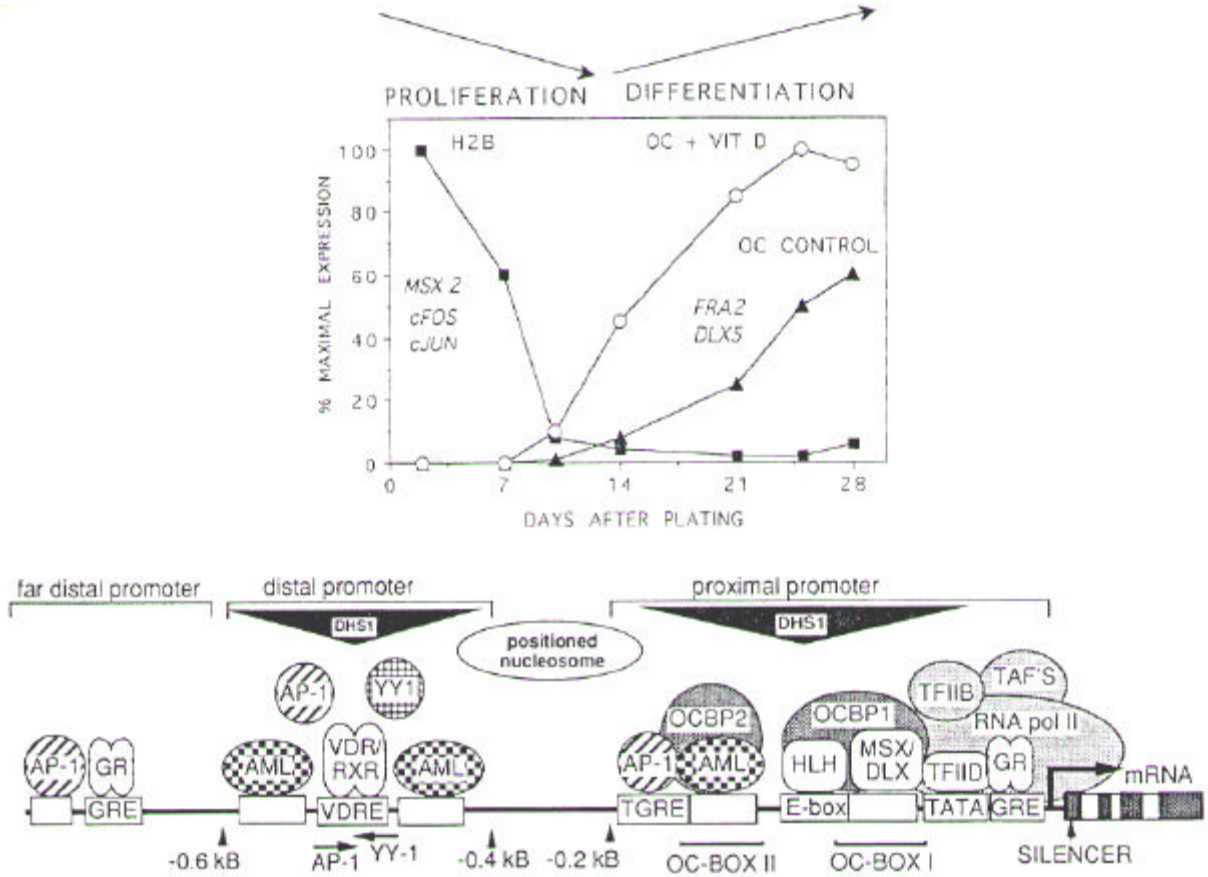

Figure 1: Expression of the osteocalcin gene and organization of promoter regulatory sequences. A. Expression of the osteocalcin gene and vitamin D enhancement during the proliferation period of the osteoblast developmental sequence. H2B mRNA is an indication of proliferative activity. B: Organization of the osteocalcin gene promoter indicating promoter regulatory domains (squares) and cognate transcription factors (circles and ovals). The OC box I and II are the primary tissue-specific transcriptional elements that bind homeodomain proteins (MSX and DLX) and/or osteoblast-specific transcription factors. Fos-jun-related proteins interact as heterodimers with AP-1 sites which overlap with or are in proximity of TGFβ (TGRE), glucocorticoid (GRE), and vitamin D_3 responsive elements (VDRE). HLH proteins bind to the E box motif contiguous with the OC box. There are three binding sites for an osteoblast-specific AML-related transcription factor which is a component of the nuclear matrix. The occupancy of OC promoter regulatory elements results in recruitment of RNA pol II, TFIIB, TFIID, and associated factors (TAFs) to the site of transcription initiation. Nuclease hypersensitive sites in the proximal and distal promoter regions are designated by filled triangles labeled DHS1.

3.2.1. Basal/Tissue Specific Factors and Sequences

A series of elements contributing to basal expression (25,26) include a TATA sequence (located at -42 to -39) and the osteocalcin box I (OC Box I), a 24 nucleotide element with a CCAAT motif as the central core. Both promoter elements are required for rendering the OC gene transcribable (27,28). OC Box 1 defines the threshold for initiation of transcription (29,30) and contributes to regulating bone tissue-specific expression of osteocalcin (31). OC Box II, which binds an AML related transcription factor spans the -138 to -130 (32,33) domain and plays a significant role in phenotype-restricted expression of the osteocalcin gene. The most compelling evidence for involvement of an AML transcription factor in control of bone tissue-specific transcription is that forced expression of AML induces osteocalcin expression in non-osseous cells where the gene is not transcribed (32,34,35). Additionally, tissue restricted (thymus, spleen, bone) expression of the AML-1, 2, and 3 genes is observed, and regulated expression of each occurs during stages of osteoblast differentiation (35). Recently, an osteoblast-specific AML/CBF-related protein with a 5' extension encoding 60 amino acids has been identified in mouse osteoblasts (36). However, the contributions of flanking sequences and cognate regulatory factors must be considered within the context of competency for binding specific mediators of the AML transcription factor family and cross talk involving proximal as well as distal regulatory domains. Because multiple sequences are operative in tissue-specific regulation opportunities are provided for stringent control of osteocalcin gene expression in bone under diverse biological circumstances.

3.2.2. Multiple Glucocorticoid Responsive Promoter Domains

Multiple glucocorticoid responsive elements (GRE) with sequences that exhibit both strong and weak affinities for glucocorticoid receptor binding have been identified in the proximal and distal promoter (37-39). Interactions of other transcription factors with the proximal glucocorticoid responsive elements have been suggested (27,37,38) further expanding the

potential of the OC gene to be transcriptionally regulated by glucocorticoids. The possibility of functional interactions of GREs with transcription factors other than glucocorticoid receptors under certain conditions should not be dismissed (40). It is reasonable to consider, based on modifications in protein-DNA interactions, that the OC gene GRE'S may be selectively utilized in a developmentally and/or physiologically responsive manner (41).

3.2.3. The Vitamin D Responsive Element

The vitamin D responsive element (VDRE) functions as an enhancer (42-46) which is tightly coupled to the changing basal levels of osteocalcin expression during osteoblast differentiation (41,47,48). Maximal activity of the VDRE is mediated by binding of the vitamin D receptor/retinoic X receptor (VDRE/RXR) heterodimeric receptor complex. However, under conditions where the osteocalcin gene is inactive or expressed at basal levels in the absence of vitamin D, the VDRE may be associated with transcription factors which include but are not restricted to fos/jun-related proteins (49,50) and YY1 (51-53). Activity of the VDRE transcription factor complex appears to be a target for modifications in vitamin D mediated transcription by other physiologic factors including, TNF-α (54) and retinoic acid (55-59).

3.2.4. Additional Osteocalcin Gene Promoter Regulatory Sequences

Additional regulatory sequences include an nuclear factor kappa B (NFkB) site also reported to be involved in regulation mediated by tumor necrosis factor alpha (TNFα) (60); a series of activating protein-1 (AP-1) sites (50,61-64), one of which mediates tumor growth factor beta (TGFβ) and in part FGF responsiveness (65,66); an E box (67) that binds helix-loop-helix (HLH) containing transcription factor complexes; a sequence in the proximal promoter that binds a multi subunit complex containing CP1/NR-Y/CBF-like CAAT factor complex (27); and an fibroblast growth factor (FGF) responsive element (27,68). Two osteocalcin gene promoter regulatory domains which exhibit recognition for transcription factors that mediate pattern formation during development are a homeodomain binding site within the OC box (27-30) and an AML-1 site (runt homology) sequence (32,34,69,70). These sequences may represent components of regulatory mechanisms that contribute to pattern formation associated with bone tissue organization during initial developmental stages and subsequently during tissue remodelling. MSX2 (28,71) and DLX5 (72) exhibit sequence-specific recognition for the same osteocalcin box element and forced expression of homeodomain factors influence osteocalcin gene transcription. Consequently, the reciprocal expression of MSX2 and DLX5 during osteoblast differentiation suggest mutual exclusive interactions with the OC box which contributes to developmental control of osteocalcin gene transcription. Involvement of other homeodomain-related genes that are expressed during skeletal development in control of osteoblast proliferation and differentiation is worthy of consideration.

3.2.5. Upstream Regulatory Sequences and In Vivo Regulation of Osteocalcin Expression

Although a majority of the responses which have to date been identified reside in the region of the promoter which spans the VDRE domain to the first exxon, upstream sequences that must be further defined, may contribute to both basal and enhancer-mediated control of transcription. Studies in transgenic mice of both human (73,74) and rat (75,76) OC gene promoters document the involvement of upstream regulatory domains in both level and specificity of expression. Additionally, intragenic and downstream regulatory sequences are viable candidates for regulatory involvement in osteocalcin gene expression (77-80).

3.2.6. Regulatory Implications of Overlapping and Contiguous Regulatory Domains

The overlapping and contiguous organization of osteocalcin gene regulatory elements (figure 1), as illustrated by the TATA/GRE, E Box/AP-1/CCAAT/homeodomain, TNFα/VDRE, YY-1/AP-1/VDRE, and TGFβ/FGF provide a basis for combined activities that support responsiveness to physiologic mediators (49,54,81-84). Additionally, hormones modulate binding of transcription factors other than the cognate receptor to non-steroid regulatory sequences. For example, vitamin D induced interactions occur at the basal TATA domain (48) and $1,25(OH)_2D_3$ upregulates MSX-2 binding to the OC box homeodomain motif as well as supports increased MSX-2 expression (27,85). It is this complexity of OC gene promoter element upregulation that allows for hormone responsiveness in relation to either basal or enhanced levels of expression.

4. NUCLEAR ARCHITECTURE SUPPORTS THE INTEGRATION OF REGULATORY SIGNALS MEDIATING BONE TISSUE-SPECIFIC TRANSCRIPTION

4.1 Parameters of Nuclear Structure Contribute to Transcriptional Control

There is a growing awareness of functional interrelationships mediating nuclear structure and function. Historically, there was a perceived dichotomy between regulatory mechanisms supporting gene expression and components of nuclear architecture. However, this parochial view is rapidly changing. The emerging concept is that both transcription and DNA synthesis occur in association with structural parameters of the nucleus. Consequently, it is imperative that the cellular and molecular mechanisms must be defined which contribute to both the regulated and regulatory relationships of nuclear morphology to the expression and replication of genes.

During the past several years, there has been an accrual of insight into the complexities of transcriptional control in eukaryotic cells. Our concept of a promoter has evolved from the initial expectation of a single regulatory sequence which determines transcriptional competency and level of expression. We now appreciate that transcriptional control is mediated by an interdependent series of regulatory sequences which reside 5', 3' and within transcribed regions of genes. Rather than focusing on the minimal sequences required for transcriptional control to support biological activity, efforts are being directed towards defining functional limits. Contributions of distal flanking sequences to regulation of transcription are being experimentally addressed. This is a necessity for understanding mechanisms by which multiple promoter elements are responsive to a broad spectrum of regulatory signals and the activities of these regulatory sequences are functionally integrated. Crosstalk between a series of regulatory domains must be understood under diverse biological circumstances where expression of genes supports cell and tissue functions. The overlapping binding sites for transcription factors within promoter regulatory

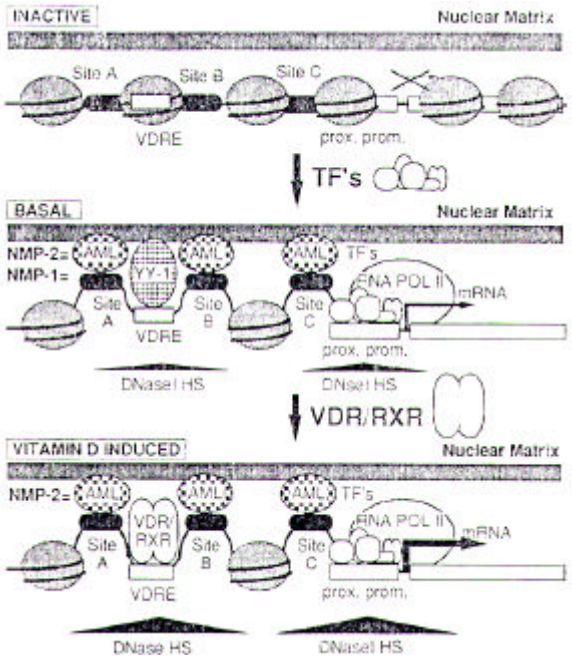

Figure 2: Schematic representation of the osteocalcin gene promoter organization and occupancy of regulatory elements by cognate transcription factors paralleling and supporting functional relationships to either: A) suppression of transcription in proliferating osteoblasts; B) activation of expression in differentiated cells, or C) enhancement of transcription by vitamin D. The placement of nucleosomes is indicated. Remodelling of chromatin structure and nucleosome organization to support suppression, basal and vitamin D induced transcription of the osteocalcin gene is indicated. The representation and magnitude of DNase I hypersensitive sites are designated by solid triangles and gene-nuclear matrix interactions are shown.

elements and protein-protein interactions which influence transcription factor activity provide further components of the requisite diversity to accommodate regulatory options for physiologically responsive gene expression.

As the intricacies of gene organization and regulation are elucidated, the implications of a fundamental biological paradox become strikingly evident. How, with a limited representation of gene-specific regulatory elements and low abundance of cognate transactivation factors, can sequence-specific interactions occur to support a threshold for initiation of transcription within nuclei of intact cells. Viewed from a quantitative perspective, the *in vivo* regulatory challenge is to account for formation of functional transcription initiation complexes with a nuclear concentration of regulatory sequences that is approximately 20 nucleotides per 2.5 yards of DNA and a similarly restricted level of DNA binding proteins.

There is a growing appreciation that nuclear architecture provides a basis for support of stringently regulated modulation of cell growth and tissue specific transcription which is necessary for the onset and progression of differentiation. Here, multiple lines of evidence point to contributions by three levels of nuclear organization to *in vivo* transcriptional control where structural parameters are functionally coupled to regulatory events. The primary level of gene organization establishes a linear ordering of promoter regulatory elements. This representation of regulatory sequences reflects competency for responsiveness to physiological regulatory signals. However, interspersion of sequences between promoter elements that exhibit coordinate and synergistic activities indicates the requirement of a structural basis for integration of activities at independent regulatory domains. Parameters of chromatin structure and nucleosome organization are a second level of genome architecture that reduce the distance between promoter elements thereby supporting interactions between the modular components of transcriptional control. Each nucleosome (approximately 140 nucleotide base pairs wound around a core complex of 2 each of H3, H4, H2 and H2B histone proteins) contracts linear spacing by seven-fold. Higher order chromatin structure further reduces nucleotide distances between regulatory sequences. Folding of nucleosome arrays into solenoid-type structures provides a potential for interactions which support synergism between promoter elements and responsiveness to multiple signaling pathways. A third level of nuclear architecture which contributes to transcriptional control is provided by the nuclear matrix (reviewed in (86,87)).

The nuclear matrix supports the structural properties of the nucleus as a cellular organelle and accommodates structural modifications associated with proliferation, differentiation and changes necessary to sustain phenotypic requirements of specialized cells (18,22-24,88-90). Regulatory functions of the nuclear matrix include but are by no means restricted to: DNA replication (91-95); gene localization (96); imposition of physical constraints on chromatin structure which support formation of loop domains (97-100); concentration and targeting of transcription factors (24,33,53,96,97,101-105); RNA processing and transport of gene transcripts (6,7,14,106-115); post-translational modifications of chromosomal proteins (116); as well as imprinting and modifications of chromatin structure (117). Association of histone acetyltransferases and histone deacetylases with the nuclear matrix is indicative of involvement in chromatin remodelling (116,118). Taken together these components of nuclear architecture facilitate biological requirements for physiologically responsive modifications in gene expression within the contexts of: 1) homeostatic control involving rapid, short-term and transient responsiveness; 2) developmental control which is progressive and stage-specific and 3) differentiation-related control which is associated with long term phenotypic commitments to gene expression for support of structural and functional properties of cells and tissues.

We are just beginning to comprehend the significance of nuclear domains in the control of gene expression. However it is already apparent that local nuclear environments which are generated by the multiple aspects of nuclear structure are intimately tied to developmental expression of cell growth and tissue-specific genes. From a broader perspective, reflecting the diversity of regulatory requirements as well as the phenotype-specific and physiologically responsive representation of nuclear structural proteins, there is a reciprocally functional relationship between nuclear structure and gene expression. Nuclear structure is a primary determinant of transcriptional control and the expressed genes modulate the regulatory components of nuclear architecture. Thus, the power of addressing gene expression within the three-dimensional context of nuclear

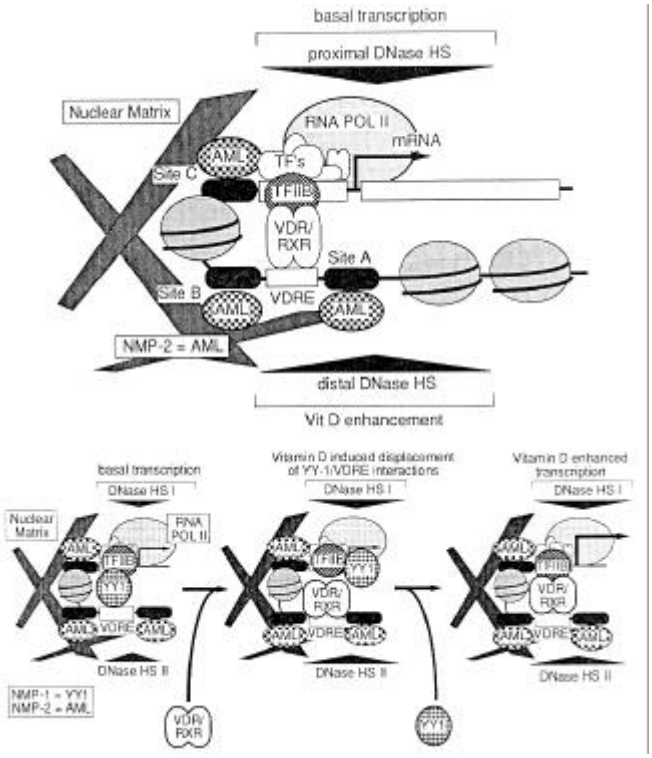

Figure 3: Three-dimensional spatial organization of the rat osteocalcin gene promoter. A model is schematically presented for the spatial organization of the rat osteocalcin gene promoter based on evidence for nucleosome placement and the interaction of DNA binding sequences with the nuclear matrix. These components of chromatin structure and nuclear architecture restrict mobility of the promoter and impose physical constraints that reduce distances between proximal and distal promoter elements. Such postulated organization of the osteocalcin gene promoter can facilitate cooperative interactions for crosstalk between elements that mediate transcription factor binding and consequently determine the extent to which the gene is transcribed. (Bottom) This model for modifications in osteocalcin gene promoter organization is consistent with protein/protein and protein/DNA interactions at the principal regulatory domains and crosstalk between regulatory elements when the gene is transcribed at basal level (left), maximally expressed following vitamin D treatment (right) and in a transition following vitamin D treatment (middle).

structure would be difficult to overestimate. Membrane-mediated initiation of signaling pathways that ultimately influence transcription have been recognized for some time. Here, the mechanisms which sense, amplify, dampen and/or integrate regulatory signals involve structural as well as functional components of cellular membranes. Extending the structure-regulation paradigm to nuclear architecture expands the cellular context in which cell-structure-gene expression interrelationships are operative.

4.2. Developmental & Steroid Hormone Modifications of Osteocalcin Gene Chromatin Structure & Nucleosome Organization

Modifications in parameters of chromatin structure and nucleosome organization parallel both competency for transcription and the extent to which the osteocalcin gene is transcribed. Changes are observed in response to physiological mediators of basal expression and steroid hormone responsiveness. This remodelling of chromatin provides a basis for the involvement of nuclear architecture in growth factor and steroid hormone-responsive control of osteocalcin gene expression during osteoblast phenotype development and in differentiated bone cells (figure 2).

In both normal diploid osteoblasts and in osteosarcoma cells basal expression and enhancement of osteocalcin gene transcription are accompanied by two alterations in structural properties of chromatin. DNase I hypersensitivity of sequences flanking the tissue-specific osteocalcin box and the vitamin D responsive element enhancer domain are observed (119-121). Together with modifications in nucleosome placement (120), a basis for accessibility of transactivation factors to basal and steroid hormone-dependent regulatory sequences can be explained. In early stage proliferating normal diploid osteoblasts when the osteocalcin gene is repressed nucleosomes are placed in the OC box and in VDRE promoter sequences; and nuclease hypersensitive sites are not present in the vicinity of these regulatory elements. In contrast, when osteocalcin gene expression is transcriptionally upregulated post-proliferatively and vitamin D mediated enhancement of transcription occurs, the osteocalcin box and VDRE become nucleosome free and these regulatory domains are flanked by DNase I hypersensitive sites (figure 2).

Functional relationships between structural modifications in chromatin and osteocalcin gene transcription are observed in response to $1,25(OH)_2D_3$ in ROS 17/2.8 osteosarcoma cells which exhibit vitamin D-responsive transcriptional upregulation.

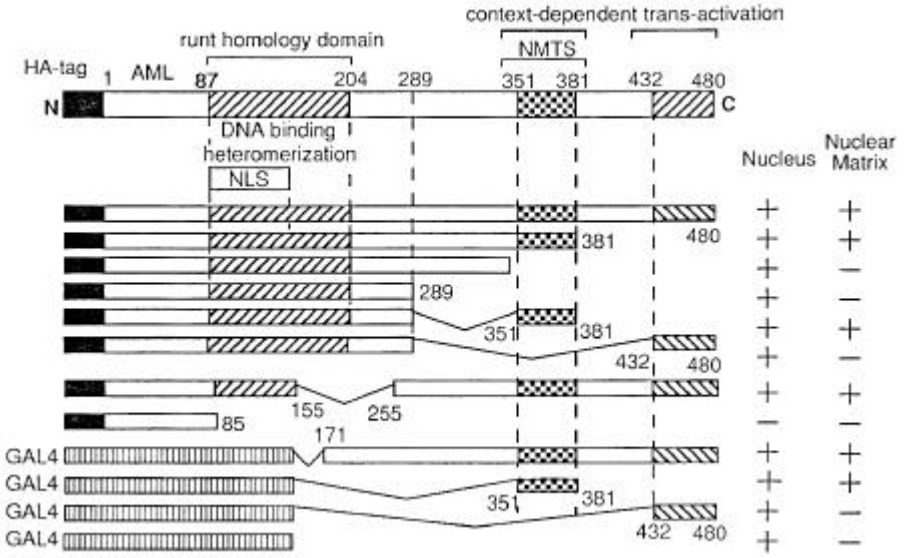

Figure 4: Delineation of the Nuclear Matrix Targeting Signal of CBFA2/AML-1B. A panel of HA-epitope tagged deletion mutants of AML-1B was assayed by immunofluorescence analysis for nuclear import (column "Nucleus") and nuclear matrix association (column "Nuclear Matrix"). C-terminal segments of AML-1B were also fused to the heterologous GAL4 DNA binding domain (aa 1-147) and analyzed similarly. The key finding is that the NMTS (aa 351-381) autonomously mediates nuclear matrix association of the GAL4 reporter protein (third line from below).

There are marked changes in nucleosome placement at the VDRE and OC box as well as DNase I hypersensitivity of sequences flanking these basal and enhancer osteocalcin gene promoter sequences (119-121). The complete absence of hypersensitivity and the presence of nucleosomes in the VDRE and osteocalcin box domains of the osteocalcin gene promoter in ROS 24/1 cells which lack the vitamin D receptor and are therefore refractory to the steroid hormone additionally corroborate these findings (119-121). These steroid hormone-responsive alterations in chromatin structure have been confirmed by restriction enzyme accessibility of promoter sequences within intact nuclei (122) and by ligation mediated PCR (LMPCR) (Montecino *et al*., in preparation) at single nucleotide resolution.

We have recently found that agents which induce histone hyperacetylation (e.g., Sodium Butyrate) promote reorganization of the nucleosomal structure in the distal region of the osteocalcin gene promoter (including the VDRE). This transition results in inhibition of the vitamin D-induced upregulation of basal transcription in ROS 17/2.8 cells. Additionally, we have established an absolute requirement for sequences residing in the proximal region of the osteocalcin gene promoter for both formation of the proximal DNase I hypersensitive site and basal transcriptional activity. Our approach was to assay nuclease accessibility (DNase I and restriction endonucleases) in ROS 17/2.8 cell lines stably transfected with promoter deletion constructs driving expression of a CAT reporter gene (123).

4.3. Contributions of the nuclear matrix to osteocalcin gene expression

Involvement of the nuclear matrix in control of osteocalcin gene transcription is provided by several lines of evidence. One of the most compelling is association of a bone-specific nuclear matrix protein designated NMP2 with sequences flanking the VDRE of the osteocalcin gene promoter (53). An additional NMP-2 binding site is a tissue-specific regulatory domain in the osteocalcin gene proximal promoter (32,53,69,70). Initial characterization of the NMP2 factor has revealed that a component is an AML-1 related transactivation protein (32,69,70). These results implicate the nuclear matrix in regulating events that mediate structural properties of the VDRE domain and basal tissue-specific gene expression.

It would be presumptive to propose a formal model for a three dimensional organization of the osteocalcin gene promoter that modulates steroid hormone responsive and developmental transcriptional control. However the working model presented in (figure 3) represents postulated interactions between OC gene promoter elements that reflect the potential for integration of activities by nuclear architecture to support transcriptional control within a three dimensional context of cell structure and regulatory requirements at the cell and tissue levels.

A role of the nuclear matrix in steroid hormone-mediated transcriptional control of the osteocalcin gene is further supported by overlapping binding domains within the VDRE for the VDR and the NMP-1 nuclear matrix protein which we have shown to be a YY1 transcription factor (51). One can speculate that reciprocal interactions of NMP-1 and VDR complexes may contribute to competency of the VDRE to support transcriptional enhancement. Binding of NMP-2 at the VDRE flanking sequence may establish permissiveness for VDR interactions by gene-nuclear matrix associations which facilitate conformational modifications in the transcription factor recognition sequences.

Taken together, these findings provide a basis for involvement of both the nuclear matrix and chromatin structure

in modulating accessibility of promoter sequences to cognate transcription factors and facilitating the integration of activities at multiple regulatory domains. Recent *in vivo* studies support functional contributions of nuclear matrix proteins to steroid hormone-mediated transcription. Overexpression of AML transcription factors which flank the

osteocalcin gene VDRE upregulates expression. In contrast, overexpression of YY1 which binds to a site overlapping the osteocalcin gene vitamin D receptor binding sequences abrogates the vitamin D enhancement of transcription and displaces VDR/RXR interactions. Functional data supporting nuclear structure-mediated crosstalk between the osteocalcin gene VDRE and the TATA domain are provided by the demonstration that the transcription factor TFIIB and the VDR cooperatively coactivate ligand-dependent transcription (124) and are partner proteins by the 2 hybrid system (125-127). Functional interrelationships between the VDRE and TATA domains under conditions where YY1 occupancy of the VDRE suppresses enhancer activity are consistent with the demonstration of mutual exclusive binding of YY1 or the VDR to the basic domain of TFIIB (52).

4.4. The Organization and Activities of AML/CBF Transcription Factors Provide a Paradigm for Interrelationships of Nuclear Architecture with Transcriptional Control

Two fundamental questions are raised with respect to functional interactions of transcription factors with the nuclear matrix. Is there a cause or effect relationship between nuclear matrix association of genes and their cognate transcription factors? What is the mechanism which targets transcription factors to the nuclear matrix. We have addressed how the AML transcription factor becomes nuclear matrix associated by functional biochemical and *in situ* immunofluorescence analysis of AML deletion and point mutations. Our results indicate that: 1) sequences required for targeting AML to the nuclear matrix reside in a 31 amino acid segment within the C-terminal that is physically distinct from the nuclear localization signal (figure 4); 2) nuclear matrix association of AML is independent of DNA binding activity; 3) the principal active and inactive splice variants of the AML transcription factor are differentially localized within the nucleus; and 4) the nuclear matrix targeting signal of AML functions autonomously. Our findings demonstrate that at least two trafficking signals are required for subnuclear targeting of AML transcription factors; the first supports nuclear import and a second mediates association with the nuclear matrix. In addition, our results suggest that loss of the C-terminal nuclear matrix targeting domain of AML, which occurs frequently in leukemia-related translocations, is functionally linked to abrogated interrelationships between nuclear structure and gene expression, characteristic of tumor cells. A basis is thus provided for addressing perturbations in the composition and/or organization of nuclear architecture that is observed in cancer. Recent results provide insight into the functional consequences of directing transcription factors to the nuclear matrix. Invoking the rationale that guilt by association is biologically relevant, it has been shown that the 31 amino acid nuclear matrix targeting sequence of the AML transcription factor targets the regulatory protein to a nuclear domain which supports transcription. Co-localization of AML with transcriptionally active RNA polymerase II has been demonstrated as well as the requirements for a functional DNA binding domain and ongoing transcription (111). Functional implications for nuclear matrix association of AML transcription factors is more directly provided by studies which establish that targeting to the nuclear matrix is obligatory for maximal transactivation activity (24).

From a general biological perspective, there is growing appreciation of sequence requirements for intranuclear targeting of steroid hormone receptors (estrogen receptor (128) and glucocorticoid receptor (129-131)) as well as ubiquitous (YY1 (132)) and selectively utilized (PIT1 (128) and PML (133)) regulatory proteins. Evidence for a nucleolar targeting domain in parathyroid hormone-related protein (PTHRP) (134) and YY1 (132) has been reported. Taken together, we are increasing our understanding of mechanisms which mediate the assembly of regulatory components to initiate and sustain transcription within the context of nuclear architecture.

5. PERSPECTIVE

We have provided an overview of how transcriptional control of the bone-specific osteocalcin gene serves as a paradigm for integration of physiological regulatory signals that are required for bone development and maintenance of functional skeletal integrity. Responsiveness of the osteocalcin gene to a broad spectrum of physiological mediators that include but are not restricted to growth factors, steroid hormones and polypeptide hormones supports stringent regulation within the context of osteoblast growth and differentiation. Regulatory mechanisms involve a cohort of tissue-specific, developmental and ubiquitous transcription factor complexes that impinge on osteocalcin gene transcription. There is a necessity to distinguish primary and secondary components of osteocalcin gene transcriptional control. Cross-talk between proximal domains of the osteocalcin gene promoter that support basal and tissue-specific activity with upstream enhancer domains that mediate steroid hormone responsiveness has been documented.

There is increasingly experimental evidence for contributions of nuclear architecture to control of gene expression. Sequences have been identified that direct transcription factors to nuclear matrix-associated sites which support transcription. Insight is thereby provided into mechanisms linked to the assembly and activities of subnuclear domains where transcription occurs. In a restricted sense, the foundation has been provided for experimentally addressing intranuclear trafficking of gene regulatory factors and control of factor association with the nuclear matrix to establish and sustain domains which are competent for transcription. In a broader context, there is growing appreciation for involvement of nuclear architecture in a dynamic and bidirectional exchange of gene transcripts and regulatory factors between the nucleus and cytoplasm, as well as between regions and structures within the nucleus.

It is difficult to arbitrarily separate nuclear structure and function or distinguish the regulated and regulatory parameters of control. The challenges we now face are to further define the targeting of transcription factors and control which reside at the level of nuclear matrix-associated acceptor sites. The result will unquestionably be further insight into fundamental processes which are involved with directing components of gene expression to specific regions within the nucleus. It would be presumptuous to propose a single model to account for the specific pathways which direct transcription

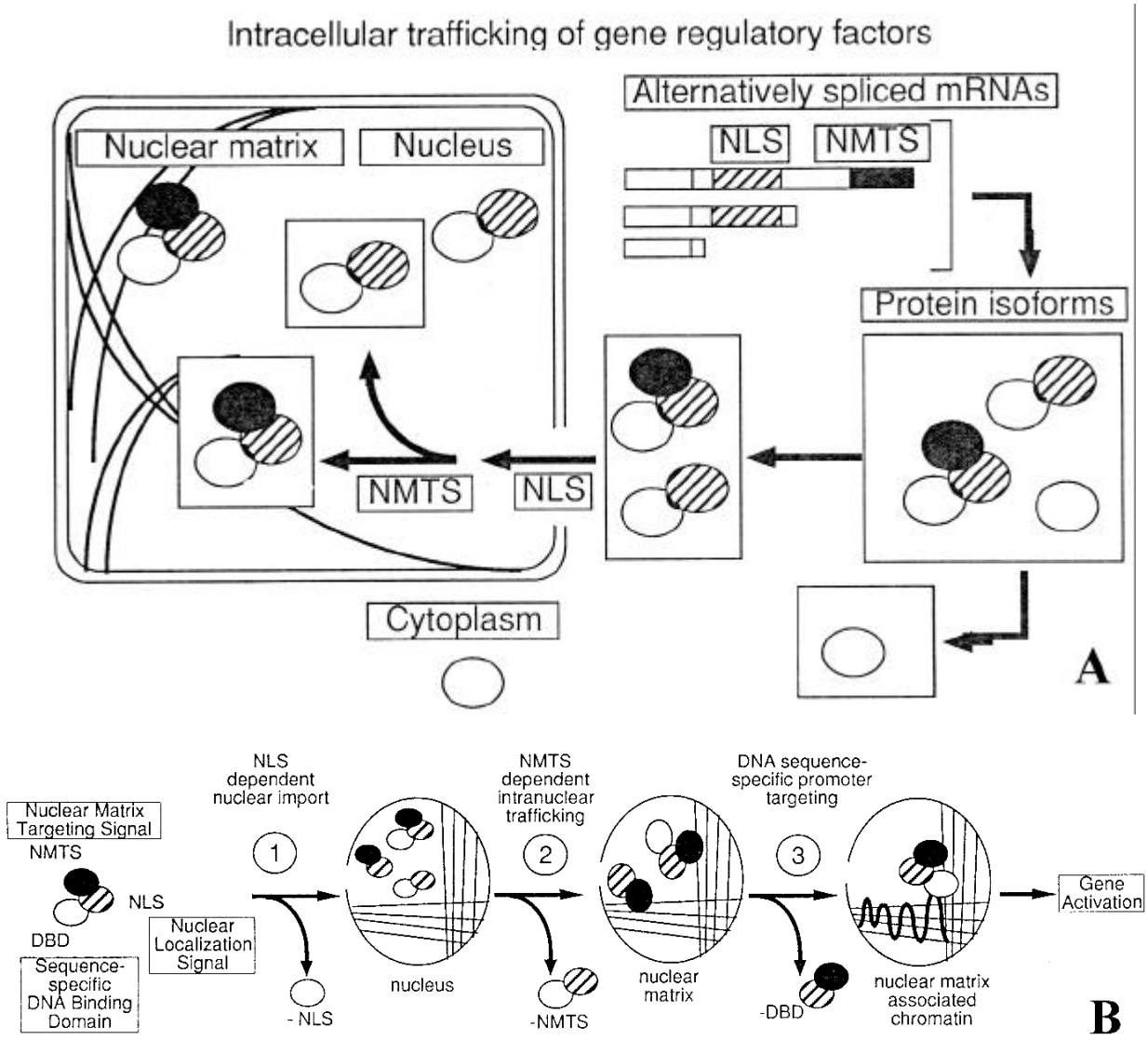

Figure 5: Intracellular trafficking of the CBFA/AML class of transcription factors supports gene activation. Panel A shows the differential intra-cellular routing of distinct CBFA/AML factors depending on presence of specific subcellular targeting signals (diagonal stripes, black filling) in protein isoforms encoded by mRNA splice variants. Panel B provides a model of the molecular sorting mechanisms which occur to support selective targeting of CBFA/AML factors to transcriptionally active domains. This involves Nuclear Localization Signal (NLS) (diagonal stripes) dependent nuclear import (Step 1), specific association with the nuclear matrix (vertical and horizontal lines) in response to the presence of a Nuclear Matrix Targeting Signal (NMTS) (black filling) (Step 2), and a requirement for a promoter recognition function of a sequence-specific DNA Binding Domain (DBD) (white filling) (Step 3) to associate with active chromatin (thick wavy line). These three steps together result in RNA pol II_0-mediated activation of AML responsive genes.

factors to sites within the nucleus that support transcription. However, findings suggest that parameters of nuclear architecture functionally interface with components of transcriptional control (figure 5). The involvement of nuclear matrix-associated transcription factors with recruitment of regulatory components to modulate transcription remains to be defined. However, working models are presented in figure 5 which serve as a framework for experimentally addressing components of transcriptional control within the context of nuclear architecture. The diversity of targeting signals must be established to evaluate the extent to which regulatory discrimination is mediated by encoded intranuclear trafficking signals. It will additionally be important to biochemically and mechanistically define the checkpoints which are operative during subnuclear distribution of regulatory factors and the editing steps which are invoked to ensure both structural and functional fidelity of nuclear domains where replication and expression of genes occur. There is emerging recognition that placement of regulatory components of gene expression must be at the right place at the right time to optimally mediate biological control. The consequences of breaches in nuclear structure-function interrelationships are observed in an expanding series of diseases, providing options for high resolution diagnosis and targeted therapy.

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