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Osteocalcin gene promoter-binding factors are tissue-specific nuclear matrix components

(DNA-binding proteins/gene expression/transcription/chromatin structure/osteoblast)

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ABSTRACT **The nuclear matrix appears to play an important role in developmental gene expression during osteoblast differentiation. To better understand this role, we examined nuclear matrix DNA-binding proteins that are sequence-specific and interact with the osteocalcin gene promoter. Multiple protein–DNA interactions involving two distinct nuclear matrix proteins occur within the 5′ regulatory sequences (nt −640 to −430). One of these proteins, NMP-1, is a ubiquitous, cell growth-regulated protein that is related to the transcription factor ATF and resides in both the nuclear matrix and the nonmatrix nuclear compartment. The other protein, NMP-2, is a cell type-specific, 38-kDa promoter factor that recognizes binding sites resembling the consensus site for the CCAAT/enhancer-binding protein C/EBP and is localized exclusively on the nuclear matrix. NMP-1 and NMP-2 each interact with two nuclear matrix protein-binding elements. These elements are present near key regulatory sites of the osteocalcin gene promoter, such as the principal steroid hormone (vitamin D)-responsive sequences. Binding in this region of the osteocalcin gene promoter suggests transient associations with the nuclear matrix that are distinct from the stable interactions of matrix attachment regions. Our results are consistent with involvement of the nuclear matrix in concentrating and/or localizing transcription factors that mediate the basal and steroid hormone responsiveness of osteocalcin gene transcription.**

Promoter-binding factors associate with DNA regulatory elements to initiate RNA transcription. This interaction cannot be simply through diffusion, since factor concentration in the nucleus is far too low. Very likely, DNA-binding factors and DNA are brought into juxtaposition by the nonchromatin scaffolding of the nucleus termed the nuclear matrix. By regulating this interaction, the nuclear matrix can play a fundamental role in transcriptional control.

The nuclear matrix is the nonchromatin nuclear substructure (1), which is organized as a proteinaceous network of polymorphic anastomosing fibers (2). Some nuclear matrix components are clearly cell- and tissue-type specific, including some matrix proteins and the heterogeneous nuclear RNA, an integral part of matrix structure (3–7). The linkage of nuclear matrix composition to cell and tissue type strongly suggests nuclear matrix participation in the regulation of gene expression. There is other evidence of nuclear matrix contribution to the regulation of gene expression. This includes the preferential association of actively transcribed genes with the matrix (8–12), the localization of RNA synthesis and pre-mRNA splicing on the matrix (13–15), and the presence of steroid receptors in the matrix fraction (16–18). Our recent studies showed that a cell cycle-regulated histone gene interacts with the nuclear matrix when actively transcribed in proliferating cells and that a sequence-specific, activating transcription factor (ATF)-related histone gene promoter-binding factor associates with the nuclear matrix (19). These results lend further support to the importance of the nuclear matrix in gene regulation.

Genomic DNA encompasses several levels of organization. The nuclear matrix mediates the formation of DNA loop domains that are anchored to matrix attachment regions (MARs) (20). These regions confer position and orientation independence on the transcriptional properties of several genes, as determined *in vitro* and *in vivo* (21–23). These stable gene attachment sites mediate long-range influences covering several hundred kilobases (24). The relationship of transient gene expression and long-term commitment for transcription of tissue-specific genes to nuclear architecture remains to be established. Both types of transcriptional control are required for the onset, progression, and maintenance of tissue-specific phenotypic properties responsive to physiological regulatory signals.

To establish the contribution of nuclear architecture to differentiation-related gene expression, we have been investigating the involvement of the nuclear matrix in transcriptional control during the progressive development of the osteoblast phenotype. Two-dimensional gel electrophoretic analysis revealed nuclear matrix proteins (25) unique to each of the three principal stages of osteoblast differentiation: (*i*) proliferation and extracellular matrix biosynthesis, (*ii*) extracellular matrix maturation and organization, and (*iii*) extracellular mineralization (26, 27). When the onset of mineralization was delayed, we observed a corresponding lag in the appearance of these stage-specific nuclear matrix proteins (25). Thus, there is a strong correlation between nuclear matrix composition and expression of genes associated with specific periods of osteoblast differentiation.

In this report we focus more directly on the role of the nuclear matrix in the developmental expression of genes during osteoblast differentiation. Specifically, we examined the contribution of nuclear matrix proteins to the promoter function of the bone-specific osteocalcin gene. We identified a 38-kDa cell type-specific promoter-binding factor that recognizes an element in the proximity of a steroid hormone receptor-binding domain of the osteocalcin gene. This DNA-binding protein resides solely in the nuclear matrix fraction of osteoblasts and osteosarcoma cells that actively express osteocalcin.

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Abbreviations: MAR, matrix attachment region; NME, nuclear matrix protein-binding element; VDRE, vitamin D-responsive element.

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MATERIALS AND METHODS

Cell Culture. Rat osteosarcoma cells (ROS 17/2.8) (28) were grown at 37°C in F12 medium (GIBCO) supplemented with 2% fetal bovine serum and 5% horse serum. Primary cultures of normal diploid osteoblasts were derived from fetal rat calvaria and were maintained as described (26). Human HL-60 promyelocytic leukemia cells were maintained in RPMI 1640 (GIBCO) containing 5% NuSerum (Collaborative Research) and 5% fetal bovine serum. Human HeLa S3 cervical carcinoma cells were grown in spinner minimal essential medium (S-MEM; GIBCO) containing 5% fetal bovine serum and 2% horse serum. Rat UMR-106 osteosarcoma cells and rat H4 hepatoma cells were cultured in MEM supplemented with 10% fetal bovine serum. The rat UMR-106 cells used in this study were found to express osteocalcin. Human MG-63 osteosarcoma cells were propagated in MEM with 5% fetal bovine serum.

Isolation of Nuclear Matrix and Nonmatrix Proteins. The nuclear matrix was isolated from cultured cells by the sequential extraction protocol of Fey *et al.* (29). Nuclear matrix proteins were solubilized as described by Fey and Penman (3). Nonmatrix nuclear proteins were prepared by 0.42 M KCl extraction of isolated nuclei according to Dignam *et al.* (30).

Analysis of Protein–DNA Interactions. Protein–DNA interactions were characterized by gel mobility-shift and methylation interference assays (31). Probes were prepared by 5′ end labeling using T4 polynucleotide kinase. Molecular weights of nuclear matrix proteins were estimated by UV crosslinking analysis (31).

RESULTS

Two Classes of Nuclear Matrix Proteins Are Associated with Osteocalcin Gene Promoter Elements. Initially, we determined the presence in the nuclear matrix of sequence-specific promoter-binding factors which interact with the osteocalcin gene 5′ regulatory sequences. We focused on two regulatory domains: (*i*) the proximal basal regulatory region (nt −141 to +41), containing both tissue-specific and glucocorticoid-responsive elements, and (*ii*) sequences residing between nt −1100 and −430, which include vitamin D response sequences and those mediating the activities of a series of transactivation factors (e.g., ATF and AP-1) (32, 33). We systematically analyzed the protein–DNA interactions in those two promoter domains by gel shift analysis using a series of restriction fragments. Comparisons were made between promoter-binding activities in preparations of nuclear matrix proteins and nonmatrix nuclear factors (0.42 M KCl-extractable transcription factors) isolated from ROS 17/2.8 osteosarcoma cells. Using this approach, we measured the association of osteocalcin gene promoter-binding factors with structural components of the nucleus.

DNA fragments encompassing nt −1100 to 1017, nt −640 to −430, and nt −141 to +23 show striking differences in the representation of protein–DNA complexes and the extent to which promoter-binding factors are part of the nuclear matrix (Fig. 1). The profiles for probes spanning nt −857 to −728, nt −728 to −640, and nt +23 to +147 have similar protein–DNA interaction patterns for both nuclear matrix and nonmatrix nuclear proteins. These results indicate that nuclear matrix proteins form complexes with the basal promoter (nt −141 to +23), the region spanning the VDRE (nt −640 to −430), and a distal domain (nt −1100 to −860) containing a putative steroid hormone receptor-binding motif.

We focused on the binding of nuclear matrix proteins to the osteocalcin gene promoter region containing the VDRE (nt −640 to −430). This steroid hormone response element mediates developmental regulation of osteocalcin gene transcription. Multiple specific protein–DNA interactions were

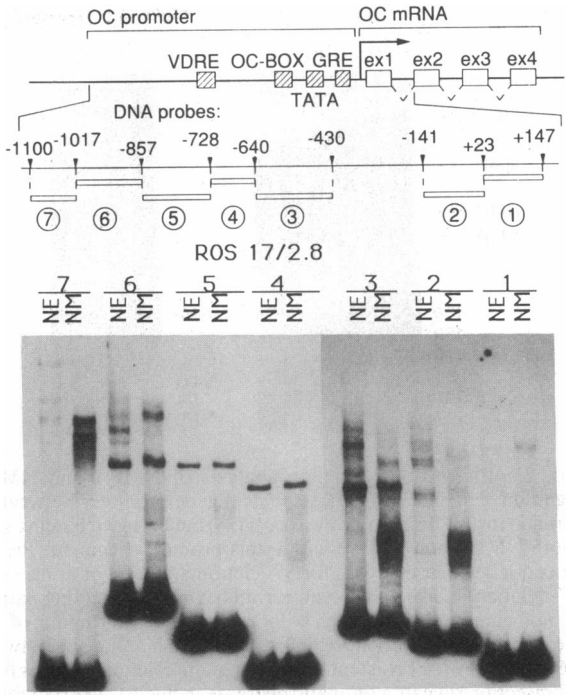

FIG. 1. Identification of protein–DNA interactions within the osteocalcin (OC) gene promoter. Shown are gel mobility-shift assays (*Lower*) using fragments of the OC promoter as probes (*Upper*) (probe numbers correspond with numbers above the lanes) to compare DNA-binding activities of nuclear matrix (NM) and nonmatrix nuclear extract (NE) proteins. VDRE, vitamin D-responsive element; GRE, glucocorticoid-responsive element; ex, exon. Nuclear matrix-specific DNA-binding activities were observed with several fragments, including nt −1100 to −1017, nt −640 to −430, and nt −141 to +23. Binding reaction mixtures (20 μl) included 75 mM KCl, 0.1 mM dithiothreitol, 500 ng of poly(dI-dC)·(dI-dC) 15% (vol/vol) glycerol, 1–2 μg of nuclear protein and 0.5 nM probe. Electrophoretic fractionation of the mixtures was performed at 4°C in a 5% polyacrylamide gel (40:1 acrylamide/*N*,*N*′-methylenebisacrylamide weight ratio) in TGE buffer (31).

observed within this fragment of the promoter (Fig. 2). NMP-1 was present in both the nuclear matrix and the nonmatrix nuclear fraction (Fig. 1) of confluent, but not proliferating, ROS 17/2.8 osteosarcoma cells (data not shown). A histone H4 gene-derived oligonucleotide (19) spanning the binding site of NMP-1 competed with the osteocalcin promoter (nt −640 to −430) for NMP-1 binding (data not shown). NMP-2 and NMP-2a were found only in the nuclear matrix fraction of ROS 17/2.8 cells (Fig. 1). Competition analysis indicates that the nuclear matrix-specific complex at the basal promoter involves the same DNA-binding activity (data not shown).

Sequence-Specific Recognition of Osteocalcin Gene Promoter Elements by Nuclear Matrix Proteins. To define the boundaries of the nuclear matrix protein recognition sites in the nt −640 to −430 fragment, we performed bidirectional deletion analysis (stairway assays; ref. 34). Two nuclear matrix protein-binding elements (NMEs) were found within this osteocalcin promoter fragment (Fig. 2). These NMEs are ≈150 bp apart, and each one interacts with both NMP-1 and NMP-2. The factor designated here as NMP-1 appears to be identical to the NMP-1 nuclear matrix protein recently isolated from HeLa S3 cells (19) (data not shown). The apparent affinity of NMP-2 for the two NMEs differs. The weaker binding site is in proximity to vitamin D and AP-1 elements, sequences integral to osteocalcin transcription (reviewed in ref. 27), whereas the other site is located further upstream.

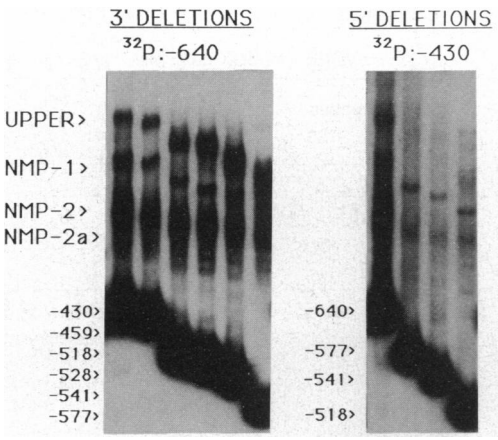

FIG. 2. Bidirectional deletion analysis of NMP-1 and NMP-2 recognition sequences in the osteocalcin gene promoter between nt −430 and −640. These stairway assays (34) indicate two binding sites for both NMP-1 and NMP-2 within this promoter segment. Probes were end-labeled at nt −640, for 3′ deletion analysis, or at nt −430, for 5′ deletion analysis. The nuclear matrix protein binding pattern does not change dramatically with progressive shortening of the probe from the 3′ direction, revealing a (distal) binding site between nt −640 and −577. Consistent with this result, deletion analysis from the 5′ direction reveals a dramatic decrease in nuclear matrix protein binding when sequences between nt −577 and −640 are deleted. However, the interactions are not completely abolished and persist with a fragment spanning nt −518 and −430, indicating a second (proximal) binding site. The prominent nuclear matrix binding activities were designated NMP-1, NMP-2, NMP-2a, and Upper.

The two NMEs differ in organization as established at single-nucleotide resolution by methylation interference analysis (Figs. 3 and 4). In the proximal NME (NME-B, nt −457 to −430) the NMP-1 and NMP-2 binding sites are nonoverlapping and contiguous with the regulatory sequences that modulate vitamin D-responsive transcriptional activity of this gene (Figs. 3 and 4). The distal domain (NME-A, nt −609 to −587) is A+T-rich (≈80%), and the NMP-1 and NMP-2 binding sites overlap (Figs. 3 and 4). An additional protein–DNA interaction (NMP-2a) was resolved and, despite a higher mobility, makes the same guanine contacts as NMP-2. This may reflect posttranslational modifications and/or protein–protein interactions.

Competition analysis (Fig. 4*B*) supports the sequence specificity of the NMP-1 and NMP-2 interactions with the rat osteocalcin promoter and clearly shows a difference in the affinity of NMP-2 for binding to NME-A and NME-B. The consensus binding sequence for NMP-2, as determined by mutational analysis (Fig. 4*C*), is 5′-RACCRCT-3′ (R = A or G). This sequence resembles the consensus binding site for the CCAAT/enhancer-binding protein C/EBP (35). The osteocalcin promoter NMP-1 binding sites (e.g., 5′-TGAGGACA-3′) exhibit sequence similarity to the ATF-related NMP-1 binding site (5′-TGACGTCC-3′) in the human histone H4 gene (19). The NMP-1 and NMP-2 binding sites in the rat osteocalcin promoter share a 5′-GNGG-3′ motif (N = A, G, C, or T) necessary for protein contact, allowing for overlapping recognition sequences.

The molecular mass of the NMP-2 protein is 38 kDa as determined by UV crosslinking analysis (Fig. 5). Consistent with the subnuclear localization of NMP-2 binding activity in the nuclear matrix, this 38-kDa protein is absent from the nonmatrix nuclear fraction.

Osteocalcin Gene Promoter-Binding Factors Are Cell Type-Specific Nuclear Matrix Components. We determined the tissue specificity of nuclear matrix protein binding to the osteocalcin promoter by gel mobility-shift analysis. The experiments used nuclear matrix proteins from a broad

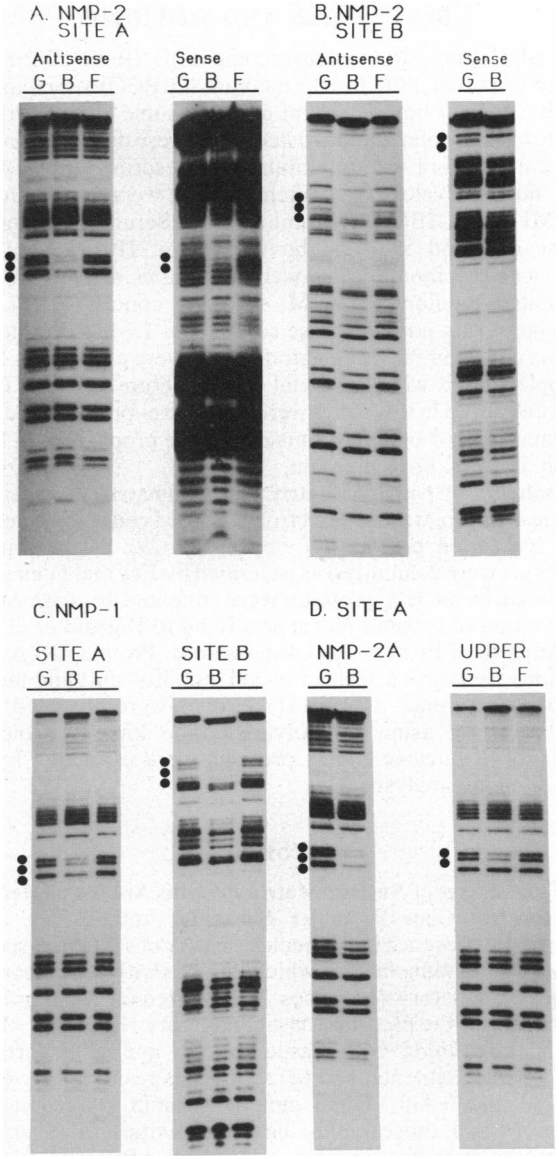

FIG. 3. Methylation interference analysis of the distal and proximal NMEs in the osteocalcin gene promoter between nt −640 to −430 (see Fig. 4*A* for schematic representation). Lanes: G, guanine sequence ladder; B, probe complexed with protein (B); F, free probe. Black circles indicate guanine or adenine methylation interference contacts. (*A* and *B*) Contacts of NMP-2 binding on both the antisense and sense strand to NME-A (nt −607 to −595, site A) and NME-B (nt −459 to −434, site B), respectively. (*C*) Contacts for NMP-1 binding to NME-A (antisense strand) and NME-B (sense strand). In each of these NMP-1 binding sites, contacts were limited to guanine residues and observed only on one DNA strand. (*D*) Contacts for nuclear matrix proteins (NMP-2a and Upper) binding to the antisense strand of NME-A. NMP-2a contacts the same three guanines as NMP-1 and NMP-2 (nt −602, −601, and −599) (*D*), with only NMP-2 and NMP-2a competing in a similar manner (data not shown). The Upper protein clearly contacts only two of these same guanine nucleotides.

spectrum of osseous and non-osseous cell types. NMP-1 is a ubiquitous binding activity that is regulated by cell growth (Fig. 6 and data not shown). In contrast, NMP-2 appears to be specific to osteoblasts and osteosarcoma cells that are actively expressing the osteocalcin gene. It should be noted that nuclear matrix proteins from all cell types examined have similar amounts of several general transcription factors (e.g., Sp1 and Oct-1; data not shown). In comparison, NMP-2 is found only in cells transcribing the osteocalcin gene. However, there does not appear to be a strict correlation between

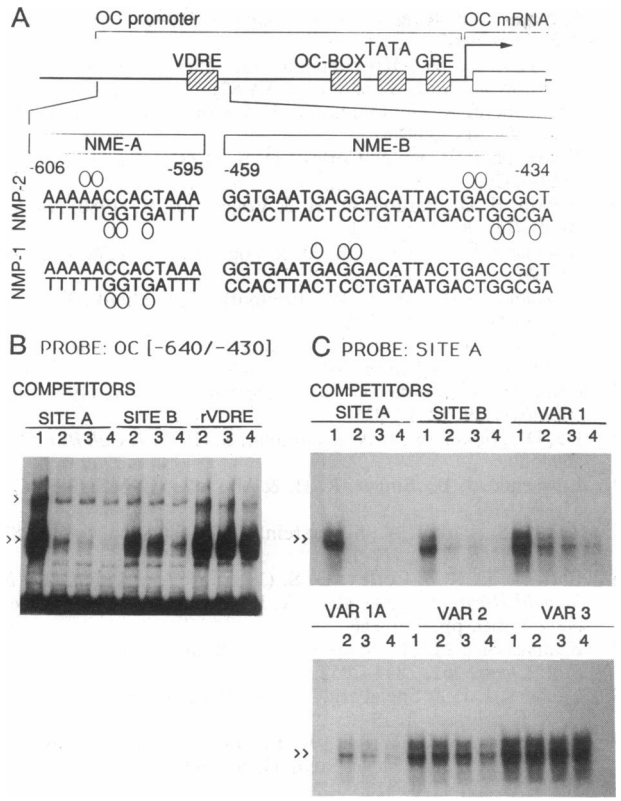

FIG. 4. (*A*) Schematic representation of the nuclear matrix protein interactions with the osteocalcin (OC) promoter. Based on methylation interference (see Fig. 3) and competition and mutation analysis (see *B* and *C*), we define two NMEs. The distal NME, NME-A (nt −609 to −587), contains overlapping recognition sites, embedded in A+T-rich sequences, for NMP-1 and NMP-2 (interference contacts indicated by ovals). This element also interacts with nuclear matrix proteins NMP-2a and Upper (not indicated). The proximal NME, NME-B (nt −457 to −430), contains two contiguous binding sites for NMP-1 (nt −453 to −446) and NMP-2 (nt −440 to −434). The NMP-1 binding site extends partially into the VDRE (the two steroid half consensus sequences span nt −459 to −445; not indicated) and AP-1 binding site (nt −457 to −451) (26). (*B*) Competition assays establish sequence specificity of the binding of NMP-1 and NMP-2 to the osteocalcin gene promoter. Mobility-shift assays were performed as in Fig. 1, with a probe spanning nt −640 to −430. The unlabeled competitor oligonucleotides used were site A (5′-GATCCCGAAAAACCACTAAAGCA-3′), spanning NME-A, site B (5′-GATCCCGACTGACCGCTCCTGCA-3′), spanning NME-B, and rVDRE (5′-CTGCACTGGGTGAATGAGGACATTACTGA-3′), spanning the rat VDRE. These oligonucleotides were present in 0, 50, 100, and 200 molar excess of probe (lanes 1–4, respectively). Note that specific competition for NMP-2 binding is more efficient with the site A oligonucleotide than with the site B oligonucleotide, suggesting that the affinity of NMP-2 for NME-A is higher than for NME-B. (*C*) Mutation analysis establishing critical nucleotides required for NMP-2 binding to NME-A. Mobility-shift assays were performed with the site A oligonucleotide as the probe. The unlabeled oligonucleotides used for competition analysis included site A and site B, as well as DNA fragments containing nucleotide variations in the NMP-2 recognition sequence: Var 1, 5′-AGAAAACCGAAAGCGCGAAAACCGAAAGCG-3′; Var 1A, 5′-TGATATACAAGAGTATCGGACCAGATTGAAAACCGAAAGCG-3′; Var 2, 5′-TGATATACAAGAGTTATCGGACCAGATTGAAAACCTGAAAGCG-3′; Var 3, 5′-TGATATACAAGAGTATCGGACCATATGTAACATAGACAGCG-3′. These oligonucleotides were present in 0, 100, 200, and 500 molar excess of probe (lanes 1–4, respectively).

NMP-2 activity and the level of osteocalcin gene expression. For example, UMR-106 and MG-63 osteosarcoma cells express levels of osteocalcin comparable to that in ROS 17/2.8 cells, but the concentrations of NMP-2 are far lower.

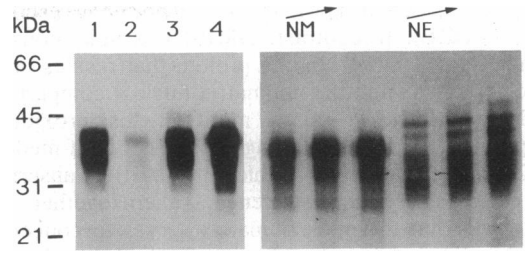

FIG. 5. Molecular weight determination of nuclear matrix proteins interacting with NME-A in the osteocalcin gene promoter. UV-crosslinking analysis was performed with a probe prepared by specific labeling of NME-A with [α-^{32}P]dGTP and bromodeoxyuridine on the antisense strand. The results demonstrate that NMP-2 has a molecular mass of ≈38 kDa. Specificity of the assay was confirmed by competition: the first four lanes contained a fixed amount of nuclear matrix protein (2.5 μg), in the absence (lane 1) of competitor or presence of a 400-fold molar excess of site A (lane 2), site B (lane 3), or a nonspecific DNA fragment (lane 4). The last six lanes contain similar increasing amounts of proteins derived from the nuclear matrix fraction (NM; ref. 2) (lanes 5–7) or nonmatrix nuclear extract fraction (NE; ref. 29) (lanes 8–10) (in each case, respectively, 0.8, 1.7, and 3.3 μg). This comparison confirms specificity of NMP-2 protein for the nuclear matrix fraction. The NMP-2 band was not observed when UV light (310 nm) exposure or proteins were omitted, or when samples were treated with proteinase K (data not shown). Molecular mass markers (indicated at left) were from Sigma.

DISCUSSION

We have identified two classes of nuclear matrix proteins that exhibit sequence-specific interactions with osteocalcin gene promoter regulatory sequences. These studies combined protein–DNA recognition analysis with characterization of nuclear matrix proteins. NMP-2 is a 38-kDa, cell type-specific, nuclear matrix-specific, osteocalcin promoter-binding protein from osteoblastic cells that recognizes a

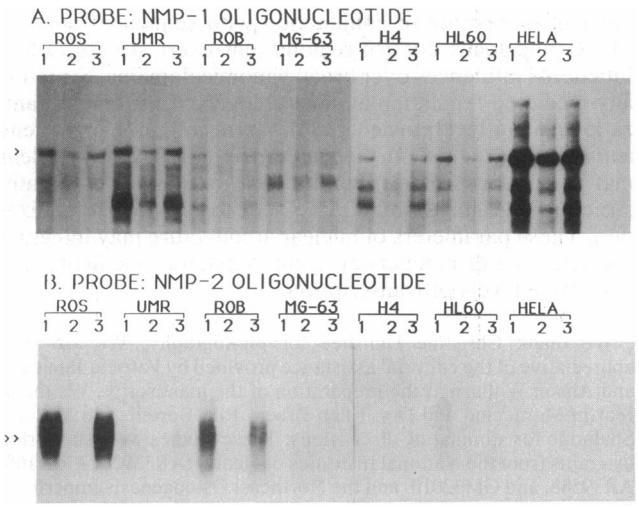

FIG. 6. Cell-type and tissue specificity of nuclear matrix proteins interacting with the osteocalcin gene promoter. Mobility-shift analysis was performed with oligonucleotide probes specific for detection of either NMP-1 (5′-GATCTGGGATTCGGCTGACGTCCATGAGAAAG-3′) (19) (*A*) or NMP-2 (site A) (*B*). NMP-1 binding activity, based on comigration and competition, is present in a variety of cell types whereas NMP-2 is restricted to rat osteoblastic cells. For each assay 1 μg of nuclear matrix proteins was used. Cell types: rat osteosarcoma ROS 17/2.8 and UMR-106 cells, primary normal diploid osteoblasts derived from rat calvaria (ROB), human MG-63 osteosarcoma, rat H4 hepatoma cells, human HL-60 promyelocytic leukemia, and human HeLa S3 cervical carcinoma cells. Each set of lanes represents a competition analysis either in the absence (lane 1) or in the presence of a 100-fold molar excess of specific (lane 2) or nonspecific (lane 3) DNA fragment.

binding site adjacent to a primary steroid hormone responsive element (VDRE). In contrast, NMP-1 is a ubiquitous, cell growth-regulated DNA-binding protein that resides in both the nuclear matrix and the nonmatrix nuclear compartment. NMP-1 recognizes ATF-related motifs in close proximity or overlapping with NMP-2 binding sites. The motif mediating NMP-2 binding strongly resembles the C/EBP transcription factor consensus binding sequence. Taken together, these data suggest that the nuclear matrix serves to concentrate and/or localize transcription factors for both basal expression and steroid hormone responsiveness.

It is necessary to understand how the association of NMP-1 and NMP-2 with the nuclear matrix contributes to bone-specific transcription of the osteocalcin gene. The packaging of DNA in the nucleus encompasses several levels of organization. The nuclear matrix, comprising the chromosome scaffold (36), as well as an internal anastomosing fibrogranular network and the lamina/pore complex (4), orders the DNA into anchored loops by means of MARs (also known as SARs and A elements) (23, 37). These elements, 200–300 nt in length and A+T-rich, are often enriched in topoisomerase II binding sites and have a high affinity for nuclear matrix proteins (38). Mapping of MAR binding sites *in vitro* showed the absence of stable and specific gene–nuclear matrix interactions within the initial 1 kb of flanking sequence surrounding the osteocalcin gene transcription start site (data not shown). This finding is consistent with the absence of extensive A+T-rich tracts in this region of the gene. Interestingly, the distal NME (NME-A, nt −609 to −587) described here resembles a truncated MAR. The presence of this sequence in the osteocalcin promoter suggests transient associations of the gene with the nuclear matrix for inducing osteocalcin gene transcription during a defined period of osteoblast development.

It remains to be established whether NMEs in the immediate vicinity of transcribed sequences are functionally independent or operate in conjunction with sequences such as MARs and locus control regions that exert structural and functional influences over broad genomic domains. Structurally mediated regulation at several levels of genome organization may reflect transient or long-term commitment to gene activity. We require further understanding of gene–nuclear matrix relationships, as well as contributions of chromatin structure and nucleosome placement to promoter organization. These parameters of nuclear architecture may integrate the activities of independent gene regulatory elements that determine levels of transcription.

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