The Tissue-Specific Nuclear Matrix Protein, NMP-2, Is a Member of the AML/CBF/PEBP2/*Runt Domain* Transcription Factor Family: Interactions with the Osteocalcin Gene Promoter†

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ABSTRACT: The nuclear matrix protein, NMP-2, was originally identified as an osteoblast-specific DNA-binding complex localized exclusively to the nuclear matrix. NMP-2 was shown to recognize two binding sites, site A (nt −605 to −599) and site B (nt −441 to −435), in the rat bone-specific osteocalcin gene promoter. This study shows that the NMP-2 binding sites A and B as well as a third NMP-2 binding site (nt −135 to −130) constitute a consensus sequence, $^{T}_{A}G^{T}_{C}GGT$, and represent an AML-1 recognition motif. AML-1 is a member of the AML transcription factor family which is associated with acute myelogenous leukemia and binds to the sequence $TG^{T}_{C}\underline{GGT}$ via its DNA-binding *runt domain*. Electrophoretic mobility shift assays reveal that a component of NMP-2 is a member of the AML/PEBP2/*runt domain* transcription factor family based on cross-competition with AML-1 consensus oligonucleotide. Limited immunoreactivity of NMP-2 with a polyclonal N-terminal AML-1 antibody and inability of the AML-1 partner protein CBF-β to form complexes with NMP-2 indicate that NMP-2 is not identical to AML-1 but represents a variant AML/PEBP2/*runt domain* protein. Western and Northern blots reveal the presence of multiple AML-related proteins and AML-1 transcripts in several osseous cell lines. Furthermore, our results indicate that AML family members may selectively partition between nuclear matrix and nonmatrix compartments. Because proteins that contain a *runt domain* are implicated in tissue-specific transcriptional regulation, our results support the concept that the nuclear matrix mediates osteoblast-specific expression of the osteocalcin gene.

The nuclear matrix is the nonchromatin structure that includes the perinuclear lamina/pore complex and the interior nuclear protein network, which resists extraction with detergent and high salt as well as digestion with nucleases (Berezney & Coffey, 1975; Fey et al., 1984). A subset of nuclear matrix components exhibits cell-and tissue-type specificity [reviewed in Fey et al. (1991) and Stein et al. (1994). The importance of the nuclear matrix in the regulation of gene expression is further supported by the following evidence: actively transcribed genes preferentially associate with the nuclear matrix (Shaack et al., 1990; Stief et al., 1989; Zenk et al., 1990); RNA synthesis and pre-mRNA splicing sites localize to the nuclear matrix (Blencowe et al., 1994; He et al., 1990; Lawrence et al., 1989); regulatory components of cell cycle progression are associated with the nuclear matrix (Dworetzky et al., 1992; Mancini et al., 1994; van Wijnen et al., 1993); steroid receptors are found in the nuclear matrix (Kumaara-Siri et al., 1986; Landers & Spelsberg, 1992; van Steensel et al., 1995); protein kinases involved in posttranslational modification associate with the nuclear matrix (Tawfic & Ahmed, 1994); and transcription factors partition in a cell type-dependent manner between the nuclear matrix and nonmatrix nuclear fractions (van Wijnen et al., 1993).

The contribution of the nuclear matrix to the progression of osteoblast phenotype development is supported by stage-specific modifications of nuclear matrix proteins during the three developmental periods of growth and differentiation in primary rat osteoblast cultures (Dworetzky et al., 1990). More recently, we have shown that nuclear matrix proteins bind to the osteocalcin gene promoter (Bidwell et al., 1993). One of these proteins, NMP-1, is ubiquitously expressed, and is found in both nuclear matrix and nonmatrix nuclear compartments (Dworetzky et al., 1992). Another protein, nuclear matrix protein-2 (NMP-2),[1] is cell type-specific and associates exclusively with the nuclear matrix. Bidwell et al. (1993) identified two NMP-2 binding sites flanking the vitamin D response element (VDRE) in the osteocalcin

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[1] Abbreviations: AML, acute myelogenous leukemia; CBF, core binding factor; EMSA, electrophoretic mobility shift assay; GRE, glucocorticoid responsive element; IF, intermediate filament; kDa, kilodalton(s); NE, nuclear extract; NM, nuclear matrix; NMP-2, nuclear matrix protein-2; nt, nucleotide; OC, osteocalcin; PAGE, polyacrylamide gel electrophoresis; PEBP, polyoma enhancer binding protein; PMSF, phenylmethanesulfonyl fluoride; SSC, sodium chloride, sodium citrate; SDS, sodium dodecyl sulfate; *rd, runt domain*; VDRE, vitamin D responsive element.

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promoter with a consensus binding sequence 5′-RACCRCT-3′ (R = A or G). This sequence resembles consensus sequences for enhancer-binding proteins C/EBP (Faisst & Meyer, 1992) and AML-1/PEBP2 (Melnikova et al., 1993; Meyers et al., 1993). PEBP2 (polyoma enhancer binding protein) is the murine counterpart of the human acute myelogenous leukemia (AML) transcription factor family. Recently, two AML-1 forms encoded by alternatively spliced transcripts have been shown to bind to the consensus sequence TG$^{T}_{C}$GGT (Meyers et al., 1993, 1995).

The AML-1 protein (27 kDa) contains a 128 amino acid DNA-binding region called the *runt domain (rd)* that shows high homology to the *Drosophila* pair-rule gene *runt* (Daga et al., 1992). The *AML-1* gene was identified because of its frequent rearrangement in acute myelogenous leukemia (Miyoshi et al., 1991; Nucifora et al., 1993). AML transcription family members which contain a *rd* include the murine AML-1 homolog PEBP2αB1 (Bae et al., 1993), AML-2 (Levanon et al., 1994), and AML-3 and its mouse homolog PEBP2αA (Levanon et al., 1994; Ogawa et al., 1993a). Deletion mutagenesis studies with AML-1 protein show that the *rd* is required for sequence-specific DNA binding and heterodimerization to CBF-β/PEBP2β which causes a dramatic increase in the DNA-binding affinity of AML-1 (Meyers et al., 1993; Ogawa et al., 1993b; Wang et al., 1993).

Proteins that contain a *rd* are important for the regulation of developmental and tissue-specific genes. For example, *runt* is an early-acting segmentation protein that regulates the expression of other *Drosophila* segmentation genes (Ingham & Gergen, 1988). The importance of *rd* proteins in determining tissue-specificity is shown by the presence of AML-1 binding sites in the T-cell-specific *lck* proximal promoter (Allen et al., 1992) and in enhancers of the T-cell receptor genes (Satake et al., 1992). In addition, Northern blots reveal tissue-specific expression of multiple forms of each *AML/PEBP2* family member in T and B cell lines using probes representing the various AML/PEB2 family members (Bae et al., 1993; Levanon et al., 1994; Ogawa et al., 1993a).

Here, we report that the rat osteocalcin promoter contains three NMP-2 binding sites which have an intrinsic AML-1 consensus sequence. We show that a component of the nuclear matrix protein NMP-2 is AML-related based on electrophoretic mobility shift assays and Western blot analysis. Also, Northern analysis demonstrates the presence of AML-1 transcripts in osseous cells and the absence of transcripts in nonosseous cells which lack the NMP-2 complex. This is the first report of an AML-related factor localizing in the nuclear matrix fraction. Our results provide supporting evidence for the concept that the nuclear matrix concentrates tissue-specific transcription factors allowing for osteoblast-specific expression of the osteocalcin gene.

MATERIALS AND METHODS

Cell Culture. Rat osteosarcoma cells (ROS) 17/2.8, ROS 24.1, and ROS 25.1 (obtained from Drs. Gideon and Sevgi Rodan, Merck Research Labs, West Point, PA) (Majeska et al., 1980) were seeded at 7×10^5 cells per 100 mm^2 plate and grown in F12 medium (GIBCO) containing 5% fetal calf serum (FCS). The rat osteogenic sarcoma cell line, UMR-106-P01 (modified by Dr. Nicola Partridge; Partridge et al., 1980), was plated at 7×10^5 cells per 100 mm^2 plate and cultured in minimal essential medium (MEM) and 10% FBS. Primary rat normal diploid osteoblasts (ROB) were derived from fetal calvaria (Aronow et al., 1990). Rat hepatoma cells, H-4 II-C3, obtained from the American Type Culture Collection, rat embryo fibroblastoid cells, R2 (Topp, 1981), and primary fetal rat lung cells (FRL) were seeded at 4×10^5 cells per 100 mm^2 plate and grown in MEM and 10% FCS.

Isolation of Nuclear Matrix and Nonmatrix Proteins. Nuclear matrix proteins were prepared by modifying the sequential extraction method of Fey et al. (1984) and solubilized as described by Fey and Penman (1988). Plated cells were washed with ice-cold phosphate-buffered saline (PBS), scraped in 1.0 mL of PBS per 100 mm plate, and pooled into a ice-cold polypropylene centrifuge tube. Cells were pelleted at 880*g* for 5 min at 4 °C. Pellets were resuspended in cytoskeleton (CSK) buffer [100 mM NaCl, 300 mM sucrose, 10 mM 1,4 piperazinediethanesulfonic acid (pH 6.8), 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, and 1.2 mM phenylmethanesulfonyl fluoride (PMSF)] to release phospholipids and soluble proteins and were then pelleted as above. Cytoskeletel proteins were extracted by resuspending the pellet in RSB−Majik buffer [100 mM NaCl, 10 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1.0% Tween-40, 0.5% deoxycholate (Na salt), and 1.2 mM PMSF], pelleted as before, and the supernatant was removed which contains polyribosomes. The remaining structure was incubated with digestion buffer [50 mM NaCl, 300 mM sucrose, 10 mM PIPES (pH 6.8), 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, and 1.2 mM PMSF] containing DNase I (100 μg/mL) and RNase A (50 μg/mL) for 20 min at room temperature on a tilt shaker. After digestion, the DNA and associated histones were released from the nucleus by adding ammonium sulfate to a final concentration of 250 mM. The nuclear matrix-intermediate filament (NM-IF) fraction consisting of <5% of the total cellular protein was recovered by centrifugation at 880*g* for 10 min at 4 °C.

The NM-IF pellet was resuspended in a disassembly buffer [8 M urea, 20 mM MES (pH 6.6), 1 mM EGTA, 0.1 mM $MgCl_2$, 1.0% 2-mercaptoethanol, and 1.2 mM PMSF] and dialyzed in bags (2000 MW cutoff) at room temperature against assembly buffer [150 mM KCl, 25 mM imidazole-hydrochloride (pH 7.1), 5 mM $MgCl_2$, 0.125 mM EGTA, 2 mM dithiothreitol, and 0.2 mM PMSF]. Dialysis was carried out for two 1 h periods in 100-fold excess assembly buffer, followed by an overnight dialysis against 200-fold excess assembly buffer. The resulting suspension was centrifuged at 150000*g* for 95 min at 20 °C using a fixed-angle rotor to pellet the reassembled IF. The supernatant containing the nuclear matrix was concentrated using Centricon tubes (Amicon, Beverly, MA). The concentrate was then dialyzed twice in the Centricon tubes with buffer D lacking glycerol [100 mM KCl, 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM PMSF]. After dialysis, glycerol was added to a concentration of 20%, and NM proteins were analyzed by EMSA or Western blots. Nonmatrix proteins (NE) were prepared by the 0.42 M KCl extraction method of Dignam et al. (1983).

Electrophoretic Mobility Shift Assay (EMSA). Protein−DNA interactions were analyzed by EMSA as previously described (Bidwell et al., 1993). Briefly, the binding reaction mixtures (20 μL) included 1−2 μg of nuclear protein, 75 mM KCl, 0.1 mM dithiothreitol (except when antibody was

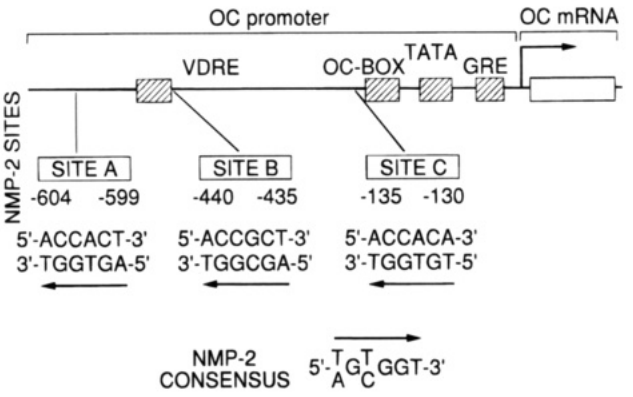

FIGURE 1: Osteocalcin promoter contains three NMP-2 binding sites. Schematic diagram of three sites in the rat osteocalcin promoter that bind NMP-2. Each site contains the sequence, GGT.

present), 200 ng of poly(dI-dC) and 50 fM probe. Probes were prepared by 5′ end labeling using T4 polynucleotide kinase. Electrophoresis was performed at 4 °C on a 5% polyacrylamide gel in TGE buffer (50 mM Tris, 380 mM glycine, and 2.1 mM EDTA, pH 8.5).

Northern Blot Analysis. RNA from various cell lines was prepared using the TRIzol reagent (GIBCO-BRL, Gaithersburg, MD). Ten micrograms of total RNA per lane was separated on a denaturing 1% agarose gel, transferred to a Zeta-Probe membrane (Bio-Rad, Philadelphia, PA), and hybridized to random-primed probes (Stratagene, La Jolla, CA). Probes contained the coding region sequences for human AML-1 (Miyoshi et al., 1991), human histone H4, pF0002 (Pauli et al., 1989), and rat osteocalcin, pOC3.4 (Lian et al., 1989). Blot hybridizations were carried out at 42 °C in 50% formamide, 5× SSC, 10× Denhardt's, 50 mM $NaPO_4$, and 100 μg/mL salmon sperm DNA. Filters were washed at 65 °C in 2× SSC, 0.1% SDS and in 1× SSC, 0.1% SDS.

Western Blot Analysis. Nuclear matrix and nonmatrix proteins (25 μg) were resolved on a 10% SDS−PAGE mini gel (Bio-Rad), electroblotted onto Hybond-ECL nitrocellulose, and visualized using ECL reagents (Amersham, Arlington Heights, IL).

Preparation of Antisera. A 17 amino acid N-terminal peptide based on the AML-1 sequence was synthesized and injected into rabbits (Meyers et al., 1993). Polyclonal antiserum was used in EMSA, while affinity-purified antiserum was prepared for Western analysis.

Purification of AML-1 and CBF-β Proteins. GST-AML-1 and GST-CBF-β were purified as previously described (Hiebert et al., 1992), and the proteins were eluted in 20 mM reduced glutathione in Tris-buffered saline (pH 7.5).

RESULTS

Three NMP-2 Binding Sites Located in the Osteocalcin Promoter Define a Shared Consensus Motif, ${}^{T}_{A}G^{T}_{C}GGT$. Previously, we identified two NMP-2 binding sites that flank the osteocalcin VDRE—site A (nt −605 to −599) and site B (nt −441 to −435)—and an additional NMP-2 interaction between nt −141 and +23 which was not further characterized at that time (Bidwell et al., 1993). Comparison of the nt −141 to +23 sequence with sites A and B revealed the presence of a third NMP-2 binding site (site C) at nt −135 to −130 (Figure 1) which was confirmed by formation of the NMP-2 complex with an oligonucleotide spanning site

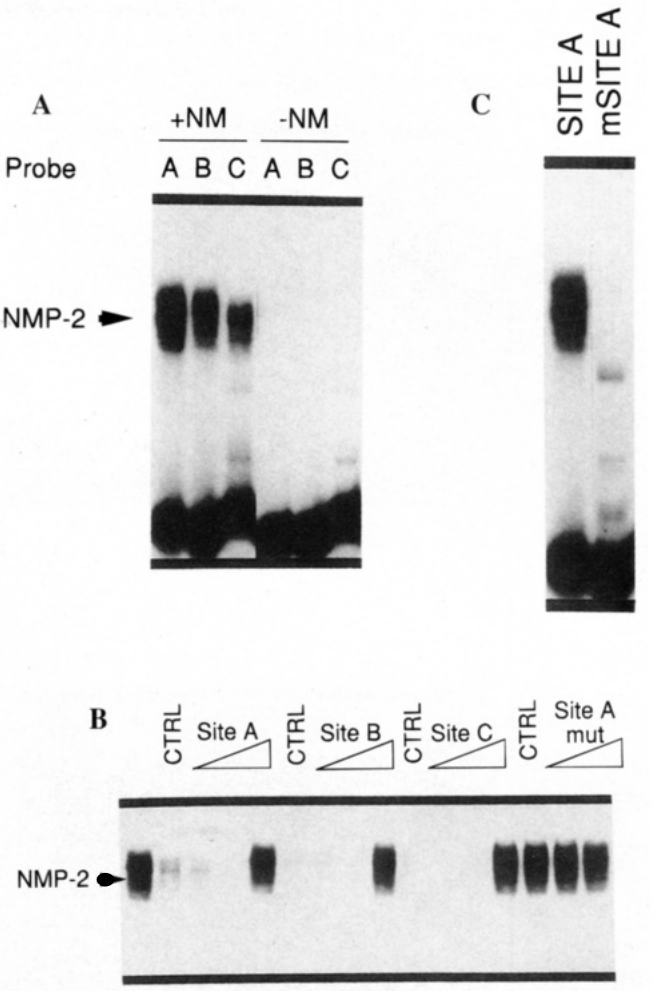

FIGURE 2: EMSA analysis of the three NMP-2 binding sites. (A) Three distinct regions of the osteocalcin promoter bind NMP-2. Probes spanning site A (5′-GATCCCGAAAAACCACTAAAGCA-3′), site B (5′-GATCCCGACTGACCGCTCCTGCA-3′), or site C (5′-CCTTCGCCCCGGCAGCTGCAGTCACCAACCACAGC-3′) were incubated with (+NM) or without (−NM) nuclear matrix proteins prepared from ROS 17/2.8 cells. (B) Competition analysis establishes sequence-specific binding of NMP-2 to three sites. Competition experiments using site A probe were performed with 20-, 40-, and 100-fold molar excesses of oligonucleotides A, B, and C and mutant site A (5′-GATCCCGAAAActaACTAAAGCA-3′). (C) The GGT core motif is essential for NMP-2 binding. Nuclear matrix proteins were incubated with oligonucleotide probes for either site A containing the GGT core motif or mutant site A (mSite A) in which this motif is altered to TAG.

C (nt −162 to −128, Figure 2A). To further confirm the identity of the three NMP-2 protein−DNA interactions, we performed cross-competition studies using site A as a probe in EMSA (Figure 2B). The NMP-2 complex was competed by unlabeled site A oligonucleotide as well as by site B and site C oligonucleotides. Comparison of the three NMP-2 sites revealed a shared consensus motif, ${}^{T}_{A}G^{T}_{C}GGT$, containing an invariant GGT trinucleotide core (Figure 1). To confirm the importance of the GGT core motif, we designed a mutation in which the GGT sequence was altered to TAG. This mutant site A oligonucleotide failed to compete with the NMP-2 protein−DNA interactions (Figure 2B). Furthermore, no NMP-2 binding activity occurred when mutant site A was used as the probe (Figure 2C). Thus, the osteocalcin promoter has three NMP-2 binding sites: site A (nt −604 to −599), site B (nt −440 to −435), and site C (nt −135 to −130).

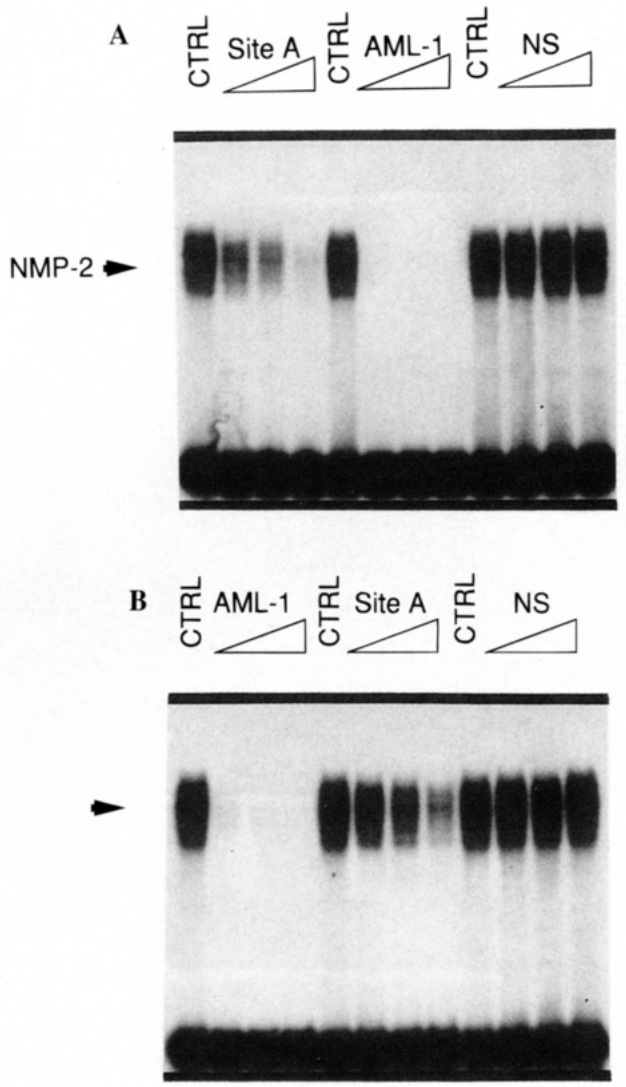

FIGURE 3: NMP-2 recognizes the AML-1 consensus binding motif, $TG^{T}_{C}GGT$. (A) Cross-competition of NMP-2 binding in EMSA using site A probe. ROS 17/2.8 nuclear matrix proteins were incubated with unlabeled competitor oligonucleotide containing site A, the AML-1 consensus binding site TGTGGT (5′-CGAGTATTGTGGTTAATACG-3′), or a nonspecific competitor, Var 3 (5′-TGATATACAAGAGTATCGGACCATATGTAACATAGACAGCG-3′). (B) NMP-2 binds the AML-1 consensus binding motif. Labeled AML-1 consensus sequence was used as probe. Cross-competition in both figure parts was with unlabeled 20-, 40-, and 100-fold molar excesses of site A, AML-1, or nonspecific Var 3 oligonucleotides.

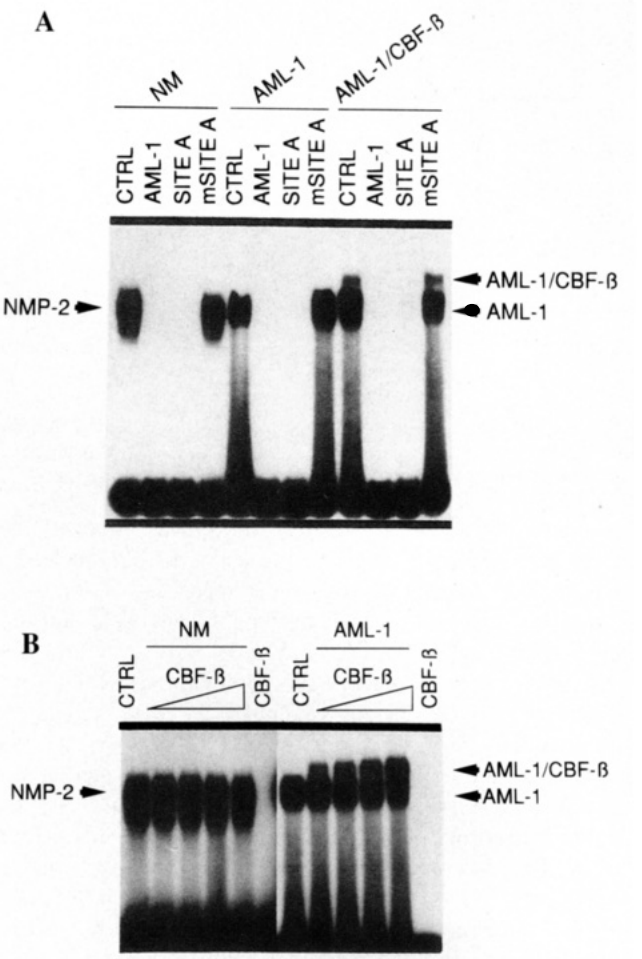

FIGURE 4: Site A contains an AML-1 recognition motif. (A) Binding of NMP-2 and purified GST-AML-1 in EMSA with site A probe. Unlabeled competitor AML-1, site A, and mutant site A oligonucleotides (100-fold molar excess) were incubated with ROS 17/2.8 nuclear matrix proteins, GST-AML-1, or GST-AML-1/GST-CBF-β. (B) Comparison of NMP-2 and AML-1 interactions with CBF-β. Increasing amounts of GST-CBF-β were incubated with constant amounts of ROS 17/2.8 nuclear matrix proteins or GST-AML-1 in EMSA using site A probe.

NMP-2 Recognizes the AML-1 Consensus Binding Motif, $TG^{T}_{C}GGT$. Recently, the consensus binding site ($TG^{T}_{C}GGT$) of AML-1, a member of the AML/PEBP2/*runt domain* transcription factor family, has been described (Meyers et al., 1993). The AML-1 consensus sequence is highly similar to the consensus NMP-2 binding site. Like NMP-2, AML-1 binding is abrogated when the invariant core nucleotides, GGT, are mutated (Meyers et al., 1993). To address whether the NMP-2 and AML consensus elements possess functionally equivalent DNA-binding properties, we examined interactions of nuclear matrix proteins with probes spanning site A and the AML-1 consensus binding sites in cross-competition studies.

Using site A as a probe (Figure 3A), NMP-2 binding activity was competed by both site A and AML-1 oligonucleotides in a dose-dependent manner. In addition, when the AML-1 consensus probe was incubated with nuclear matrix proteins (Figure 3B), we observed a complex with migration properties indistinguishable from those of NMP-2. This complex was competed by the same set of oligonucleotides used to characterize NMP-2 binding. Notably, there are interesting quantitative differences in competition efficiency, suggesting that the AML-1 consensus binding site has a higher affinity for NMP-2 than does the osteocalcin site A binding site. Thus, cross-competition results establish that NMP-2 can bind the AML-1 consensus sequence.

NMP-2 Is Related to the AML/PEBP2/Runt Domain Transcription Factor Family. To further define the relationship between the nuclear matrix protein NMP-2 and the *runt domain* AML-1 proteins, we compared the ability of NMP-2 and purified recombinant GST-AML-1 to interact with the AML-1 partner protein, CBF-β, in EMSA. CBF-β is a non-DNA-binding subunit that when heterodimerized to AML-1 increases the affinity of AML-1 for DNA binding (Ogawa et al., 1993b). Competition analysis with wild-type and mutant site A oligonucleotides revealed that both AML-1 and AMl-1/CBF-β specifically bind to site A (Figure 4A), which establishes site A as an AML-1 recognition motif. Notably, purified GST-CBF-β was capable of direct interac-

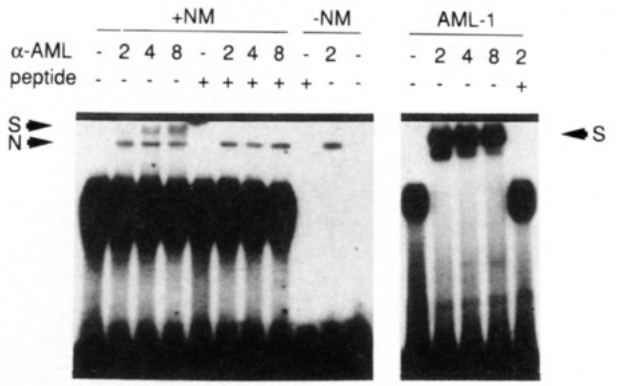

FIGURE 5: A component of NMP-2 is AML-related. An antibody directed against AML-1 complexes with NMP-2 in EMSA. Rabbit antiserum was raised to an N-terminal peptide of AML-1 that exhibits homology to other AML family members. α-AML-1 was incubated with (+NM) or without (−NM) ROS 17/2.8 nuclear matrix proteins in the presence of site A probe (left panel). α-AML-1 (2, 4, 8 μL) generated a supershift complex (upper arrow, S) and a nonspecific lower band (lower arrow, N). The nonspecific band is observed in the absence of nuclear matrix proteins. Only the specific band was abolished by addition of the antigenic N-terminal AML-1 peptide (20 μg). The right panel shows formation of an α-AML-1/GST-AML-1 supershift complex (arrow, S) which is blocked by addition of antigenic AML-1 peptide.

tion with purified GST-AML-1, while GST-CBF-β did not efficiently form complexes with nuclear matrix proteins (Figure 4B). This result suggests that NMP-2 is not identical to AML-1 but may represent a variant AML/PEBP2/*runt domain* protein with identical DNA-binding properties that is incapable of heterodimerizing with CBF-β (Ogawa et al., 1993b; Wang et al., 1993).

AML-1 is the prototypical member of the expanding AML class of gene regulators which exhibit high amino acid homology at their N-terminus both between species and between analogous genes (Levanon et al., 1994). To further address the relationship of NMP-2 to the AML transcription factor family, we examined the immunoreactivity of NMP-2 using a polyclonal antibody directed against the N-terminal peptide of AML-1 (aa 1−17). Figure 5 (left panel) shows that α-AML-1 interacted with NMP-2 in EMSA to produce a ternary antibody/protein/DNA complex (supershift) in a concentration-dependent manner using site A probe. The specificity of the supershift complex was confirmed by competition with the immunogenic N-terminal AML-1 peptide. This result establishes that NMP-2 is immunologically related to the AML/PEBP2/*runt domain* family of proteins. Parallel binding reactions using the same antibody concentrations and purified GST-AML-1 protein revealed that GST-AML-1 was completely converted into a supershift complex (Figure 5, right panel). In contrast, excess α-AML-1 produced only a limited amount of supershift complex when nuclear matrix proteins were used. The limited cross-reactivity of the AML-1 antibody provides further support that NMP-2 is not identical to AML-1 but is a member of the AML/PEBP2/*runt domain* transcription factor family.

Comparison of Nuclear Matrix and Nonmatrix Proteins. Previously, we have shown that ROS 17/2.8 nonmatrix nuclear protein preparations contain a DNA-binding activity that interacts with site A and migrates differently from nuclear matrix NMP-2 activity in EMSA and UV-cross-linking analysis (Bidwell et al., 1993). To determine the presence of AML-1 and related proteins in ROS cells, we

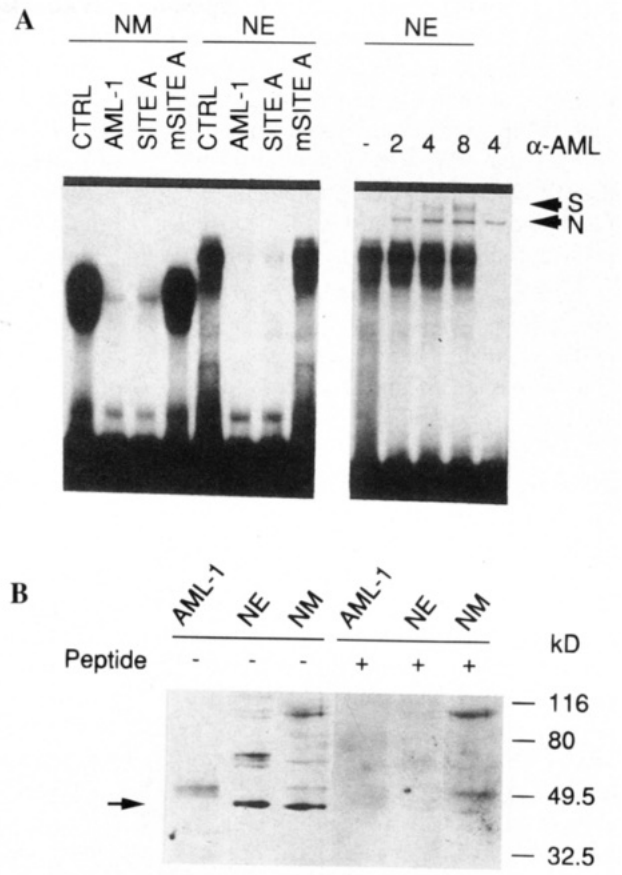

FIGURE 6: Properties of the proteins present in nuclear matrix and nonmatrix protein fractions. (A) ROS 17/2.8 nuclear matrix and nonmatrix protein complexes exhibit differential mobilities in EMSA. Nuclear matrix (NM) and nonmatrix proteins (NE) were incubated with unlabeled competitor oligonucleotides (20-fold molar excess) in the presence of site A probe (left panel). Treatment of nonmatrix proteins with α-AML-1 (right panel) resulted in formation of a supershift (S) and a non-specific band (N). (B) Western blot analysis of ROS 17/2.8 nuclear proteins. Blots containing GST-AML-1, nuclear matrix, and nonmatrix proteins were incubated with affinity-purified N-terminal α-AML-1 with (+) or without (−) competitor N-terminal antigenic AML-1 peptide. The arrow indicates a major band (45 kDa) found in both NM and NE that was specifically recognized by the AML-1 antibody. Molecular mass markers (indicated at right) were from Amersham (Arlington Heights, IL).

analyzed both ROS 17/2.8 nuclear matrix and nonmatrix proteins by EMSA using site A probe (Figure 6A). In comparison to nuclear matrix proteins, nonmatrix proteins mediated the formation of a slower migrating complex. Both nuclear matrix and nonmatrix proteins were competed by AML-1 and site A oligonucleotides. Nonmatrix proteins incubated with α-AML-1 and site A probe produced a specific supershift that is qualitatively and quantitatively similar to that observed with nuclear matrix proteins (Figure 5). The limited cross-reactivity of the AML-1 antibody in nonmatrix protein preparations further suggests that AML-related proteins are present in both subnuclear compartments of osteoblasts.

For additional characterization of AML-related proteins in ROS 17/2.8 cells, we analyzed both nuclear matrix and nonmatrix proteins by Western blots (Figure 6B). Both nuclear fractions contained a prominent 45 kDa protein that was recognized by an affinity-purified antibody made against an N-terminal peptide of AML-1. The specificity of the antibody/protein interactions in both preparations was con-

firmed by competition with N-terminal AML-1 peptide. The presence of peptide abolished detection of the 45 kDa protein as well as purified GST-AML-1 protein. It is noteworthy that several other higher molecular weight proteins present in the matrix and/or non-matrix protein fractions also showed competition. The competition results in Figure 6B indicate that ROS 17/2.8 cells may contain multiple AML family members that reside in both nuclear matrix and nonmatrix nuclear compartments.

Relationship of NMP-2 and Expression of AML-Related Transcripts. Since NMP-2 is an osteoblast-restricted complex and AML-1 expression has been studied primarily in hematopoietic cell lines (Levanon et al., 1994; Miyoshi et al., 1991), we examined several rat osseous and nonosseous cell lines using an AML-1 cDNA probe containing the *runt domain*. Northern blot analysis (Figure 7A) demonstrated that the osteosarcoma cell line, ROS 17/2.8, and primary rat osteoblasts (ROB) expressed two major transcripts, 3.8 and 6.2 kb, which have been reported in other cell types (Bae et al., 1993). Interestingly, of the nonosseous cell lines examined, both R2 rat embryo fibroblastoid and FRL fetal rat lung cells expressed low levels of AML-1 transcripts, in contrast to H4 rat hepatoma cells. To further establish a linkage of AML to NMP-2, EMSA was performed with these same cells using a site A probe (Figure 7B). As expected, nuclear matrix preparations from ROS 17/2.8 and several closely related cells lines, ROS 24.1 and ROS 25.1. The low levels of NMP-2 binding activity in nonosseous R2 cells and the absence of such activity in H4 nuclear matrix fractions suggest a relationship between NMP-2 activity and the presence of AML transcripts.

DISCUSSION

Recent evidence suggests that partitioning of transcription factors into nuclear matrix and nonmatrix compartments plays a role in the transcriptional regulation of gene expression (Stein et al., 1994; van Steensel et al., 1995; van Wijnen et al., 1993). In this study, we characterize the osteoblast-restricted nuclear matrix factor, NMP-2, which binds to three sites (A, B, C) in the rat osteocalcin promoter. Our studies demonstrate that NMP-2 is a member of the AML/PEBP2/*runt domain* transcription factor family but is not the prototypical AML-1. This is the first study to show that a member of this family localizes to the nuclear matrix.

Limited immunoreactivity with the NMP-2 complex was observed with an antibody directed against the 17 N-terminal amino acids of the prototypical human AML-1. This suggests that NMP-2 is antigenically related but not identical to the murine AML-1 homolog, PEBP2αB1 (49 kDa), which shows 100% homology to AML-1 (27 kDa) at its N-terminus (1−27 aa; Bae et al., 1993). Recently, a longer isoform of AML-1, AML-1B, which has an N-terminal extension and a molecular mass of 55 kDa has been described (Meyers et al., 1995). The presence of a rat AML family member having a modified amino terminus in the NMP-2 complex may explain the limited immunoreactivity using an AML-1 antibody directed to the N-terminal domain. Alternatively, NMP-2 may be an osteoblast-specific member of the AML/PEBP2/*runt domain* transcription factor family. Interestingly, Meyers et al. (1993) found that the AML-1 antibody did not cross-react with complexes from Saos-2 human

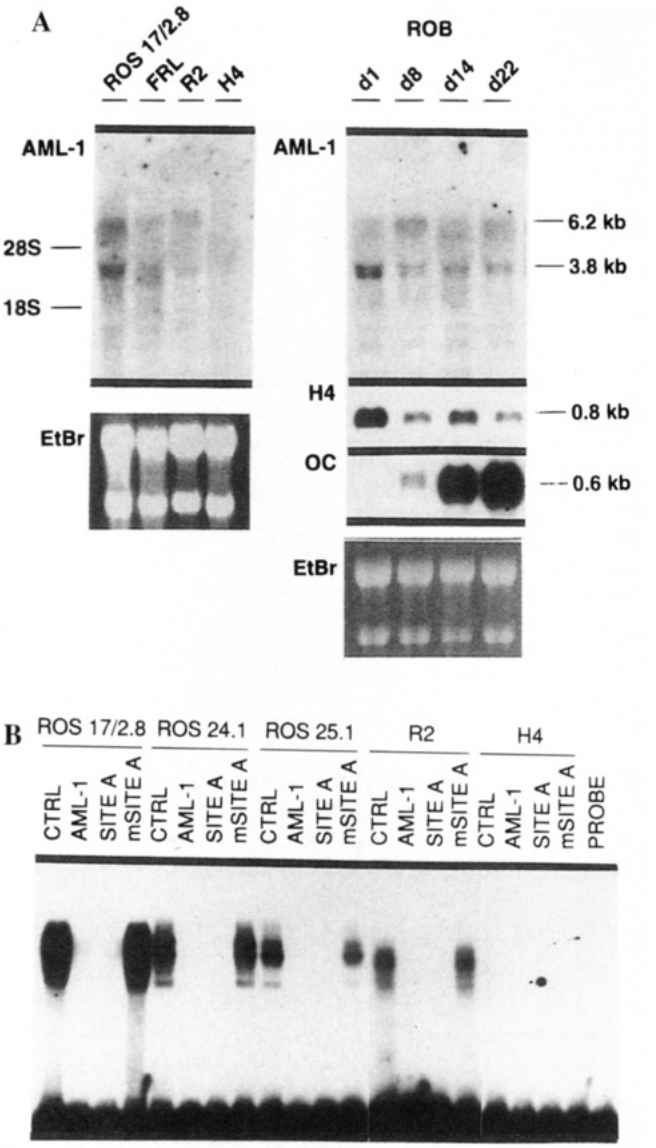

FIGURE 7: Representation of NMP-2 and AML-related transcripts in osseous and nonosseous cells. (A) Northern blot analysis of AML-related transcripts in various rat cells. Ten micrograms of total RNA from various rat cell lines (left panel) and from ROB cells at different days of development (right panel) was examined by Northern blot analysis. Blots were hybridized with cDNA probes containing coding region sequences for human histone H4, rat osteocalcin (OC), or full-length human AML-1. Ethidium bromide (EtBr) staining of 18S and 28S ribosomal RNA is shown in the lower panel. (B) Representation of NMP-2 in various cells lines. EMSA was performed on nuclear matrix proteins prepared from a variety of cell lines with site A probe using a 100-fold molar excess of unlabeled competitor oligonucleotides (AML-1, site A, and mutant site A). EMSA exposure times for R2 and H4 cells were twice that of the ROS cell lines. Cell types: rat osteosarcoma ROS 17/2.8, ROS 24.1, and ROS 25.1; rat osteogenic sarcoma UMR-106; primary normal diploid osteoblasts derived from rat calvaria (ROB); rat H4 hepatoma cells; fetal rat lung cells (FRL); and rat embryo fibroblastoid cells (R2).

osteosarcoma whole-cell extracts using the AML-1 consensus probe but did cross-react with extracts from several human T-cell and leukemia cell lines. Thus, bone cells may produce a unique member of the AML transcription factor family. The exact identity of the AML-related protein found in the nuclear matrix of rat osteosarcoma cells is under investigation.

We observed that nuclear matrix and nonmatrix proteins that bind the NMP-2 site exhibited differential mobilities in

EMSA (Figure 6A) which suggests that the NMP-2/protein−DNA complex exhibits unique properties. Western blot analysis (Figure 6B) revealed a similar immunoreactive 45 kDa protein in both subnuclear compartments though different minor proteins were also observed, particularly in the nuclear matrix fraction. This suggests that AML-related proteins associated with each compartment may be modified differently, possibly for different functions.

Another contributing factor to the different properties of NMP-2 compared to nonmatrix complexes could relate to the association of AML family members with partner proteins. AML/PEBP2 proteins typically form a heterodimer complex consisting of a tissue-restricted α subunit and a more ubiquitous β subunit which together exhibit a higher affinity for DNA than does the α subunit alone (Wang et al., 1993). Recently, Lu et al. (1995) have shown that the PEBP2β subunit, normally found in the cytoplasm, can be translocated into the nucleus by a truncated PEBP2α subunit. Our finding that NMP-2 does not form a heterodimer with purified CBF-β (Figure 4B) further suggests that ROS 17/2.8 cells contain a variant AML family member. Alternatively, the differences in mobility between subnuclear compartments may be explained by posttranslational modifications of the AML-related protein or by a conformational change brought about by the denaturation/renaturation steps of the nuclear matrix protocol.

Our finding that NMP-2 DNA-binding activity is AML-related provides new insights into developmental control mechanisms in osteoblasts. In *Drosophila*, *runt domain* proteins play a critical role in regulating development and tissue-specific gene expression since *runt* is an early-acting segmentation protein (Ingham & Gergen, 1988). Recently, Sakakura et al. (1994) have shown that a truncated AML-1 protein containing the *runt domain* can inhibit growth and induce differentiation in leukemic cells. Though previous studies have focused on AML/PEBP2/*runt domain* expression in hematopoietic and muscle cells (Levanon et al., 1994; Satake et al., 1995; Zhu et al., 1994), we have found AML-related transcripts in a variety of rat osteoblast cell lines, though not in the rat H4 hepatoma cell line. Comparison of EMSA results with Northern analysis shows that all osteoblastic cell lines that express AML-1 transcripts also contain NMP-2 activity (Figure 7). We have previously demonstrated that NMP-2 is a tissue-specific complex which interacts with three regulatory domains of the osteocalcin promoter: two that flank the VDRE and one located in the proximal promoter (Bidwell et al., 1993). Ducy and Karsenty (1995) have recently reported that ROS 17/2.8 nuclear extracts bind to a site in the mouse osteocalcin promoter that appears to be equivalent to the NMP-2/AML-1 consensus sequence at site C (nt −135 to −130). They demonstrated that a multimeric construct of their site confers tissue-specific expression. Their findings are consistent with our description of a tissue-specific DNA-binding activity associated with the NMP-2/AML-1 consensus motif. Thus, proteins containing a *runt domain* not only play an important role in the differentiation of leukemic cells but also may be involved in osteoblast differentiation.

Nuclear matrix protein−DNA interaction sites can function at several levels to regulate gene expression, e.g., by modifying structural constraints of the promoter to facilitate the association of transcription factors with DNA-binding sequences. Interestingly, analysis of the chromatin structure of the osteocalcin gene promoter shows one DNase I hypersensitive site in the distal promoter near NMP-2 binding sites A and B which flank the VDRE and another hypersensitive site located between nt −170 and −70 which spans the NMP-2 binding site C (Montecino et al., 1994). The intensity of hypersensitivity is positively correlated with osteocalcin gene expression. This is consistent with the concept of the contribution of nuclear structure to gene expression (Stein et al., 1994) and further supports the involvement of the AML-related NMP-2 complex in regulating osteocalcin at the level of modifications in gene structure. Our findings suggest that the tissue-specific nuclear matrix protein, NMP-2, is a unique AML-related protein that mediates osteoblast-specific expression of the osteocalcin gene.

ADDED IN PROOF

At a recent Human Genome Mapping Workshop (1995), the AML-1, AML-2, and AML-3 proteins were assigned the names CBFα2, CBFα3, and CBFα1, respectively.

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