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Groucho/TLE/R-esp proteins associate with the nuclear matrix and repress RUNX (CBFα/AML/PEBP2α) dependent activation of tissue-specific gene transcription

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SUMMARY

The Runt related transcription factors RUNX (AML/CBFα/PEBP2α) are key regulators of hematopoiesis and osteogenesis. Using co-transfection experiments with four natural promoters, including those of the osteocalcin (OC), multi drug resistance (MDR), Rous Sarcoma Virus long terminal repeat (LTR), and bone sialoprotein (BSP) genes, we show that each of these promoters responds differently to the forced expression of RUNX proteins. However, the three RUNX subtypes (i.e. AML1, AML2, and AML3) regulate each promoter in a similar manner. Although the OC promoter is activated in a C terminus dependent manner, the MDR, LTR and BSP promoters are repressed by three distinct mechanisms, either independent of or involving the AML C terminus, or requiring only the conserved C-terminal pentapeptide VWRPY. Using yeast two hybrid assays we find that the C terminus of AML1 interacts with a Groucho/TLE/R-esp repressor protein. Co-expression assays reveal that TLE proteins repress AML dependent activation of OC gene transcription. Western and northern blot analyses suggest that TLE expression is regulated reciprocally with the levels of OC gene expression during osteoblast differentiation. Digital immunofluorescence microscopy results show that TLE1 and TLE2 are both associated with the nuclear matrix, and that a significant subset of each colocalizes with AML transcription factors. This co-localization of TLE and AML proteins is lost upon removing the C terminus of AML family members. Our findings indicate that suppression of AML-dependent gene activation by TLE proteins involves functional interactions with the C terminus of AML at the nuclear matrix in situ. Our data are consistent with the concept that the C termini of AML proteins support activation or repression of cell-type specific genes depending on the regulatory organization of the target promoter and subnuclear localization.

Key words: Groucho, TLE, AML, Cbfa, RUNX, Transcriptional control

INTRODUCTION

The Runt related transcription factors [RUNX*/acute myelogenous leukemia (AML)/core binding factor (CBF)/polyoma enhancer binding protein 2 (PEBP2)] represent essential gene regulatory proteins that control lineage commitment and development (Speck et al., 1999). RUNX proteins are encoded by one of three distinct genes which we will refer to as AML1, AML2 and AML3. Each α subunit contains a phylogenetically conserved DNA binding domain (i.e. Runt homology domain), as well as a series of transcriptional regulatory domains and subcellular trafficking signals (Bruhn et al., 1997; Kanno et al., 1998; Kitabayashi et al., 1998; Levanon et al., 1998; Meyers et al., 1996; Petrovick et al., 1998; Speck et al., 1999; Thirunavukkarasu et al., 1998; Westendorf et al., 1998; Yagi et al., 1999; Zeng et al., 1997). A number of somatic chromosomal defects involving the AML1 (RUNX1/CBFA2) locus are functionally linked to acute myelogenous leukemia (Golub et al., 1995; Meyers et al., 1993; Mitani et al., 1994; Nucifora and Rowley 1995; Song et al., 1999; Tenen et al., 1997; Yergeau et al., 1997). Null mutation of AML1 or the heterodimeric partner protein CBFβ results in severe defects in hematopoiesis (Okuda et al., 1996; Speck et al., 1999; Speck and Stacy 1995; Wang et al.,

*The nomenclature committee of the Human Genome Organization has recently adopted the following designations for Runt-related transcription factors: RUNX1 (AML1/CBFA2/PEBP2αB), RUNX2 (AML3/CBFA1/PEBP2αA), and RUNX3 (AML2/CBFA3/PEBP2αC).

1996a,b). Genetic ablation of the AML3 (RUNX2/CBFA1) gene causes developmental defects in osteogenesis (Komori et al., 1997), and hereditary mutations in the AML3 gene are linked to specific ossification defects as observed in cleidocranial dysplasia (Otto et al., 1997). Thus, proteins encoded by distinct AML gene subtypes perform specialized functions during tissue development and differentiation in vivo.

Different AML subtypes are involved in transcriptional control of phenotype-specific genes. For example, the hematopoiesis related genes encoding granulocyte-macrophage colony-stimulating factor, interleukin 3, T-cell receptor β, and multi-drug resistance 1 (MDR1) have been shown to be regulated by AML1 (Cockerill et al., 1996; Frank et al., 1995; Lutterbach et al., 1998a,b; Sun et al., 1995; Uchida et al., 1997). Viral long terminal repeats (LTRs) are known targets of AML factors and represent the primary determinants of the tissue specificity and oncogenic potential of murine leukemia-related retroviruses (Martiney et al., 1999; Zaiman and Lenz, 1996). The expression of bone cell-specific genes including osteocalcin (OC), bone sialoprotein (BSP) and collagenase type 3 are regulated by AML3 (Banerjee et al., 1996, 1997; Ducy et al., 1997; Jimenez et al., 1999), which is the most abundant AML related protein in osteogenic and chondrogenic cell lineages. The AML2 (RUNX3/CBFA3) protein has been shown to regulate the mouse germline Ig α promoter (Hanai et al., 1999; Shi and Stavnezer, 1998), but the physiological role of AML2 in tissue development has not been established. Although distinct AML factors regulate different sets of genes in vivo, there are molecular similarities in the three AML protein subtypes.

In this study, we show that the AML1, AML2 and AML3 proteins have shared gene regulatory properties, and that the C termini of AML proteins support both promoter context-dependent activation and repression. We used the C-terminal domain of AML1 as a bait to screen a cDNA library in a yeast two-hybrid system designed to identify transcriptional regulators. One of the interacting proteins we characterized is a member of the Groucho/TLE/R-esp class of proteins (Stifani et al., 1992). In both *Drosophila* and mammals, Groucho proteins have been shown to interact genetically with Runt and repress activation of genes targeted by Runt (Aronson et al., 1997; Levanon et al., 1998; McLarren et al., 2000; Thirunavukkarasu et al., 1998). We demonstrate that the human proteins TLE1 and TLE2 associate with the nuclear matrix and colocalize with the AML proteins in the nucleus. Our studies suggest that the C-termini of AML proteins interact functionally with TLE proteins at the nuclear matrix in situ to suppress transcription of AML responsive genes.

MATERIALS AND METHODS

Promoter and expression constructs

Constructs containing the rat osteocalcin (–1,097/+23) or chicken bone sialoprotein (–620/+25) promoters fused to the chloramphenicol acetyl transferase reporter have been reported previously (Banerjee et al., 1997; Yang and Gerstenfeld, 1997). The human multiple drug resistance –1 promoter (–137/+30; Thottassery et al., 1997) and the entire RSV long terminal repeat were fused to the luciferase reporter. Each of these promoters contains one or more AML-responsive motifs. The expression constructs encoding the wild-type AML1, AML1Δ289, AML2 and AML3 are as reported earlier (Banerjee et al., 1997; Meyers et al., 1996; Zeng et al., 1997).

AML3Δ501 was constructed by digesting the pcDNA3.1 plasmid carrying mouse PEBP2αA1 cDNA (Lu et al., 1995) with *Eco*RI-*Xba*I to remove sequences encoding amino-acids 501-513, followed by blunting and ligation. AML3Δ376 was generated by truncating after amino acid 376 by PCR, or pCMV5, using the following oligonucleotide as forward primer 5′CCGCTCGAGGTCGACT-CACTCAGTGAGGGATG 3′ and 5′GGTGTCCCGCCTCAGAAC 3′ as reverse primer. The BsgI-*Xho*I digested PCR fragment was then ligated into BsgI-*Xho*I digested pCDNA3.1-AML3. Sequences at the junction of these constructs were confirmed by the dideoxy chain termination method.

TLE1 and TLE2 coding regions were amplified from pBluescript vectors carrying TLE1 and TLE2 cDNAs by polymerase chain reaction (PCR) using the following primers: TLE1 forward primer, 5′GTAATGGATCCAGTATGGGGTTCCCGCAGAGCCGGCACCC-G3′; TLE1 reverse primer, 5′GCCATACTCGAGCTAGTAGATG-ACTTCATAGACTGTAGC3′; TLE2 forward primer, 5′GCAGCGG-ATCCAGCATGGAATACCCCCAGGGAAGGCAC3′; TLE2 reverse primer, 5′GCCTGGCTCGAGTCAGTAGACCACCTCATACAC3′. The PCR fragments of TLE1 and TLE2 were digested with *Bam*HI-*Xho*I and cloned into *Bam*HI-*Xho*I digested pcDNA3.1 (Invitrogen Corporation, Carlsbad, CA). The TLE proteins expressed from these constructs carry the Xpress epitope at the amino terminus. AML and TLE expression constructs used in this study were subjected to the dideoxy chain termination method. Furthermore, [^{35}S]methionine-labeled in vitro translated proteins were prepared with the TNT coupled reticulocyte lysate system (Promega Corp., Madison, WI). Expression plasmids (1.2 μg) of AML1, AML2, AML3, AML3Δ501, AML3Δ376, TLE1 and TLE2 were used as templates. The translated products (3 μl) were subjected to SDS-PAGE, and autoradiography revealed products of the expected sizes (data not shown).

Two-hybrid screen

The PCR product coding for amino acids 345 to 480 of human AML1 was used as the target module (‘bait’) and subcloned in frame with the coding region of the LexA DNA binding domain in pEG202 (Ausubel et al., 1997a,b). An oligo dT primed yeast two-hybrid cDNA library (developed by Genome Therapeutics Corp., Waltham, MA) was prepared from ROS 17/2.8 osteosarcoma cells. The cDNAs were inserted between the *Sal*I and *Not*I sites of construct pOP46 which produces a fusion protein containing the nuclear localization signal (NLS) of SV40 T antigen and the GAL4 activation domain. The yeast reporter genes LacZ or histidine synthase contain 8 tandem repeats of the LexA binding site. The screening procedures to identify positive clones were performed according to published protocols (Ausubel et al., 1997a,b).

Cell cultures and transient transfection

HeLa and NIH 3T3 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Rat osteosarcoma (ROS 17/2.8) cells were grown in F12 supplemented with 5% fetal calf serum. HeLa and ROS 17/2.8 cells plated in 6-well, 35 mm plates were transiently transfected with 2.5 μg of promoter-CAT (BSP or OC) and 0.75 μg of either CMV empty vector or AML and/or Xpress tagged TLE expression constructs using 7 μl of SuperFect Transfection reagent (Qiagen Inc, Valencia, CA). RSV-luciferase plasmid (100 ng) was included as an internal control for transfection efficiency. Cells were harvested 24-36 hours post transfection and assayed for CAT activity. The data were normalized to luciferase values obtained from the same samples. For MDR1 and LTR promoters, NIH 3T3 cells were transfected by SuperFect Transfection reagent as recommended by the manufacturer. Briefly, 5 μg of promoter-luciferase, 1 μg of either CMV empty vector or AML expression construct, 1 μg of Renila luciferase plasmid as an internal control, and 15 μl of Superfect reagent were added to cells. Luciferase

activity was determined by using the Dual Luciferase Reporter Assay System (Promega Inc, Madison, WI). All transfections were performed in replicates of six and the experiments were repeated 3-4 times. Each result is reported as the mean with standard deviation.

RNA analysis and western blots

Total cellular RNA was isolated from normal rat fetal calvarial osteoblasts as described previously (McCabe et al., 1995). Cells at different stages of differentiation were washed and scraped in PBS. Frozen cell pellets from each time point were thawed and processed together. RNA was extracted as previously described (Chomczynski and Sacchi 1987), electrophoresed in 1% agarose gel and transferred to Hybond-N^+ membrane (Amersham Pharmacia Biotech, Arlington Heights, IL) for northern blot analyses. Blots were hybridized with random primed (Prime-It kit; Stratagene, La Jolla, CA), ^{32}P-labeled cDNA probes for TLE1 (*Bam*HI-*Xho*I fragment) and osteocalcin at 42°C overnight. The blots were washed and subjected to autoradiography. Western blots were performed on whole cell lysates from ROS 17/2.8 cells and rat osteoblast cultures at various stages of differentiation. A total of 30 μg protein was resolved by 12% SDS-PAGE and transferred to Trans-Blot (Bio-Rad, Hercules, CA). The blots were incubated with 1:30 dilution of rat monoclonal 'panTLE' antibody (Stifani et al., 1992) to detect the endogenous levels of TLE proteins. The blot was stripped and reprobed with 1:100 dilution of mouse monoclonal antibody to B23 (provided by P.-K. Chan, Baylor College of Medicine, Houston, TX) as a control for equal loading.

Immunofluorescence microscopy

Transfected or untransfected ROS 17/2.8 and HeLa cells grown on coverslips (Fisher Scientific, Springfield, NJ) were extracted according to procedures documented previously (Choi et al., 1998; Tang et al., 1999; Zeng et al., 1998). In brief, for whole cell preparation, cells were rinsed twice with ice-cold PBS and fixed in 3.7% formaldehyde in PBS for 10 minutes on ice. After rinsing twice with PBS, the cells were permeabilized in 0.1% Triton X-100 in PBS, and rinsed twice with PBSA (0.5% bovine serum albumin [BSA] in phosphate buffered saline [PBS]) followed by antibody staining. For cytoskeletal (CSK) preparation, cells were extracted in ice-cold CSK buffer (100 mM NaC1, 0.3 M sucrose, 10 mM pipes, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, pH 6.8) containing RNase inhibitors (2 mM vanadyl ribonucleoside complex or 40 U/ml RNasin) twice for 15 minutes each to remove cytosol, followed by 10 minute fixation in 3.7% formaldehyde in CSK buffer (without VRC). Cells were washed in PBSA before antibody staining. For nuclear matrix intermediate filament (NMIF) preparations, cells were extracted 2 times for 15 minutes each with ice-cold CSK buffer and then chromatin was digested by two consecutive 30 minute incubations at 25°C with the addition of digestion buffer (DB, same as CSK buffer except the NaCl concentration is 50 mM rather than 100 mM) containing 50 U/ml RNase-free DNase I (Roche Molecular Biochemicals, Indianapolis, IN). The NMIF was obtained by further extraction for 10 minutes on ice with stop solution (digestion buffer supplemented with 0.25 M ammonium sulphate). Subsequently, cells were fixed in ice-cold digestion buffer containing 3.7% formaldehyde and subjected to antibody staining.

Antibody staining was performed by incubating whole cell, CSK and NMIF preparations with a (1:300) dilution of either affinity purified rabbit polyclonal antibody against AML1, AML2 or AML3 (Meyers et al., 1996) or a (1:500) dilution of mouse monoclonal anti-Xpress antibody to label the epitope tag of TLE proteins (Invitrogen, Sand Diego, CA) for 1 hour at 37°C. Coverslips were rinsed four times with PBSA before addition of secondary antibody. Secondary antibodies were either Alexa 488 goat anti-rabbit or Alexa 568 goat anti-mouse (Molecular Probes, Eugene, Oregon). Cells were incubated with 1:800 dilution of the secondary antibody at 37°C for one hour and then were washed four times with PBSA. The cells were then stained with 4′,6-diamidino-2-phenylindole DAPI (5 μg/μl) for 5 minutes and washed once with PBSAT (0.1% Triton in PBSA) and twice with PBS. Immunostaining of cell preparations was recorded using an epifluorescence or confocal microscope attached to a CCD-camera, and the digital images were analyzed with the Metamorph and TCS-NT software programs.

Image restoration and deconvolution

Immunofluorescence microscopy of ROS 17/2.8 or HeLa cells transiently co-transfected with AML and TLE proteins was performed using a Zeiss Axioplan 2 microscope (Zeiss Inc., Thornwood, NY) equipped with epifluorescence filters and a charge-coupled device (CCD) camera interfaced with the MetaMorph Imaging system (Universal Imaging, Media, PA). Images of positive cells were acquired with a ×100 PlanApo objective with a 1.4 numerical aperture at 0.25 micron intervals (13-20 sections per cell) through focus (in the Z plane) with 56 nm per pixel (x,y), and restored to subvoxels of 28×28×50 nm as described (Carrington et al., 1995).

Fluorescent beads (189 nm) were imaged under the same optical conditions as the cell, to determine the microscope-camera point spread function (PSF). Background values from a region in the cytoplasm and camera noise (dark current) were subtracted (<5% of total). The dye density was then estimated by the non-negative function, *f*, that minimizes

$$\| \text{g-PSF} \times f \|^2 + \alpha \iiint |f|^p ,$$

where g is the measured cell image, α is a smoothing parameter, and p is the convergence. Images were reconstructed according to the above algorithm with the following range of parameters: $\alpha=10^{-4}$, $p=10^{-3}$ with 100-300 iterations. The middle section of representative cells is shown in the figures.

RESULTS

Promoter context-dependent suppression and activation by the AML1, AML2 and AML3 transcription factors

The Runt related transcription factors AML1, AML2 and AML3 each activate phenotype-specific genes containing the core binding factor consensus motif 5′ TGTGGTT and display considerable amino acid similarity and conservation of functional domains (Fig. 1A).

Co-transfection experiments illustrate that several AML responsive gene promoters (e.g. MDR, LTR, BSP) are not induced by forced expression of AML1, but instead are repressed (Fig. 1B). Basal activity of these promoters in different cell types has been documented (Martiney et al., 1999; Thottassery et al., 1997; Yang and Gerstenfeld 1997). For comparison, the OC gene can be stimulated by AML1 under analogous experimental conditions (Fig. 1B). The gene-specific activation and suppression of promoter activity are also observed with forced expression of the AML2 and AML3 subtypes (Fig. 1B). Thus, physiological regulation of AML dependent gene expression in vivo is not necessarily related to intrinsic differences in AML subtypes, but may be dictated in part by tissue-specific differences in the levels of AML proteins, as well as transcriptional co-factors that stimulate or repress activity of AML proteins.

The AML transcription factors are functionally bimodular with an N-terminal DNA binding domain and a C-terminal transcriptional regulatory domain (Fig. 1A). To address the role of the C terminus in activation or repression, we carried out co-transfection experiments with a panel of AML dependent genes and expression vectors encoding full length (1-480) or

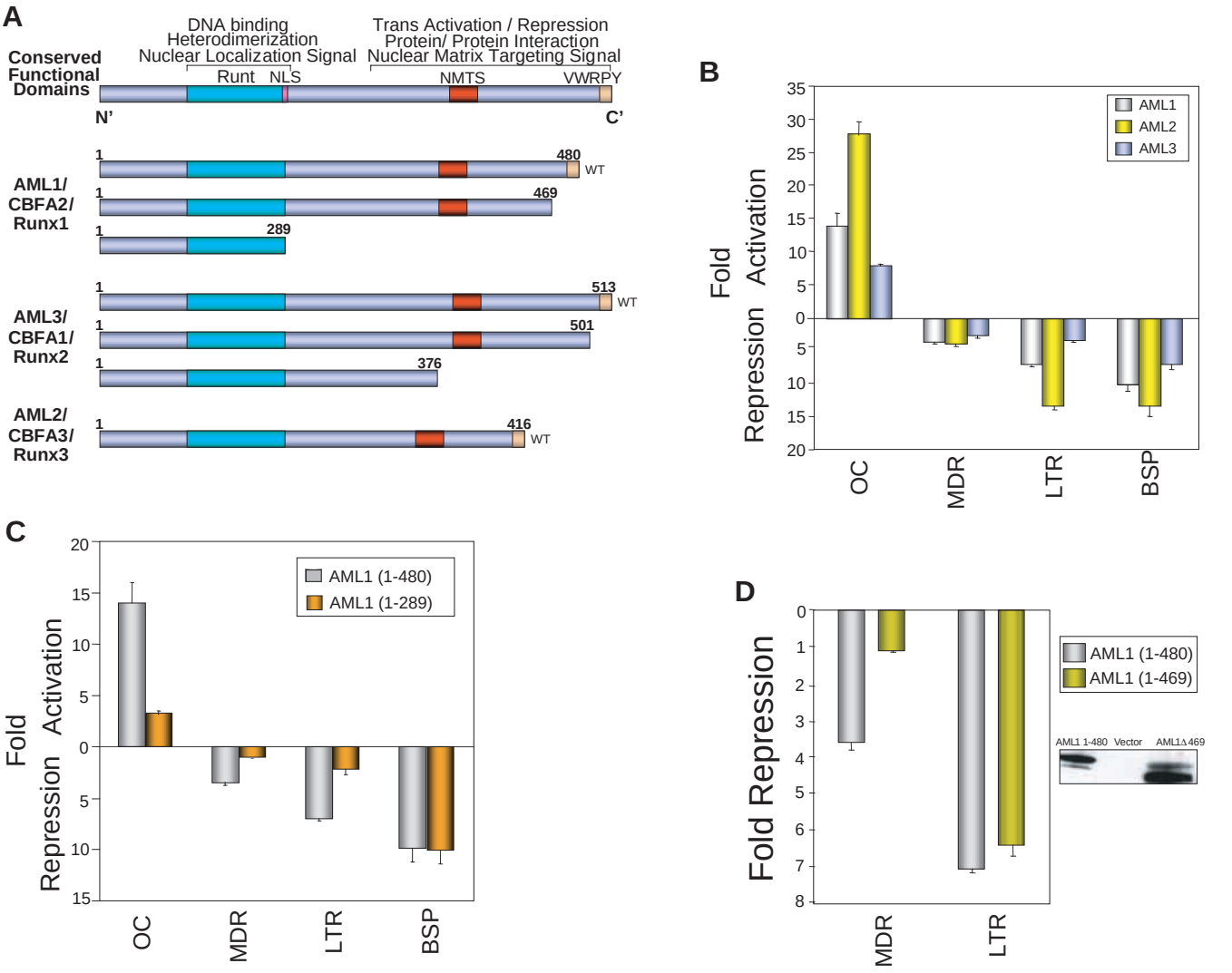

Fig. 1. Three distinct mechanisms mediate promoter-context dependent repression of AML responsive genes. (A) Schematic illustration of wild-type [WT; AML1(1-480), AML3(1-513), and AML2(1-416)] and deletion mutant constructs of AML family members. Highly conserved domains of the AML transcription factor family are denoted in the top line. The DNA binding and CBF interacting domain (Runt), nuclear localization signal (NLS), nuclear matrix targeting signal (NMTS), and conserved VWRPY penta-peptide are indicated. (B) All three AML members repress the promoter activities of MDR1, RSV LTR, and BSP genes, but not the Osteocalcin gene. AML family members were co-transfected separately with promoter-reporter constructs of OC and BSP gene in HeLa cells, MDR1 and RSV LTR genes in NIH3T3, respectively (see Materials and Methods). Similar results were obtained in ROS17/2.8 cells (data not shown). Each bar represents least square mean plus or minus standard error of the mean, LS + s.e.m. (n=12). (C) The C terminus is required for activation of OC and repression on MDR1 and LTR, but not for suppression of BSP gene promoters. Full-length AML1 (1-480) or C terminus deleted (1-290) constructs were co-transfected with indicated promoter-reporter constructs in HeLa and NIH3T3 as above. Each bar represents LS mean + s.e.m. (n=12). (D) VWRPY dependent and independent suppression of LTR and MDR1 genes. Either full-length AML1 (1-480) or a construct with the last ten amino aids deleted (1-469) was co-transfected with MDR1 or RSV LTR promoter-reporter constructs in NIH3T3 cells. The western blot shows the expression level of WT (1-480) and mutant (Δ469) protein.

C-terminally truncated (1-289) AML1 proteins. The data indicate that repression of the MDR and LTR promoters, as well as activation of the OC gene, is observed with the full length but not the truncated AML1 protein (Fig. 1C). In contrast, repression of the BSP promoter occurs with both the full length and truncated proteins (Fig. 1C). Hence, only a subset of AML1 dependent genes requires the C terminus of AML1 for suppression, while the N-terminal region containing the DNA binding domain suffices for suppressing some genes (e.g. BSP).

AML factors contain a C-terminal VWRPY motif that is phylogenetically conserved among all vertebrate AML subtypes and in the *Drosophila* Runt protein (Aronson et al., 1997). To assess the role of this pentapeptide motif in repressing the MDR1 or LTR promoters, we performed co-transfection assays with plasmids encoding either the wild-type AML1 protein or a mutant protein which has a C-terminal deletion of the VWRPY motif (Fig. 1D). We find that the LTR is repressed independent of the presence of the VWRPY motif, whereas repression of the MDR1 promoter requires the

segment containing the VWRPY motif. Thus, at least two distinct domains in the C terminus can mediate gene suppression by different mechanisms.

In summary, Fig. 1 shows four AML responsive promoters each with mechanistically distinct responses to the forced expression of either AML1, AML2 or AML3. The OC promoter is activated in a C terminus dependent manner, whereas the MDR, LTR and BSP promoters are repressed. The BSP promoter is repressed independent of the C terminus of AML1, while the MDR and LTR promoters are repressed by AML1 using VWRPY-dependent and independent mechanisms, respectively.

The multifunctional C terminus of AML1 interacts with a member of the Groucho/TLE/R-esp class of repressor proteins

Although several proteins have been identified that interact with the C-terminal region of AML1 (Ahn et al., 1998; Bruhn et al., 1997; Hanai et al., 1999; McLarren et al., 2000; Petrovick et al., 1998; Westendorf et al., 1998), the full complement of co-factors that can modulate AML function in distinct cell types remains to be established. To characterize proteins capable of interacting with the multifunctional C terminus of AML proteins, we performed yeast two hybrid screens with a rat osteoblast cDNA library using a fusion protein containing the LexA DNA binding domain and the C-terminal 345-480 amino acids of AML1 as the target of protein/protein interactions ('bait'; Fig. 2A).

Our primary screen yielded several clones that activate the β-GAL reporter gene. Sequence analysis of a cDNA clone designated H5a revealed that it encodes a truncated R-esp1 protein (Fig. 2B). R-esp1 is a member of the Groucho/TLE/R-esp class of repressor proteins which have been shown to interact functionally with Runt homology proteins (Aronson et al., 1997; Imai et al., 1998; Levanon et al., 1998; Thirunavukkarasu et al., 1998).

Groucho/TLE/R-esp proteins repress activation of the bone-related osteocalcin gene by AML1, AML2 or AML3 in osseous and non-osseous cells

In vivo gene activation of AML responsive promoters by specific AML protein isoforms occurs only in distinct cell types at particular developmental stages. For example, transcriptional induction of OC gene expression is only observed in bone cells at late stages of osteoblast differentiation and involves increased DNA binding activity of bone-specific AML3 protein isoforms (Banerjee et al., 1997). However, promoter activity of the bone-specific osteocalcin gene can be upregulated by AML1, AML2 or AML3 in cell culture assays using non-osseous cells (see Fig. 1B).

One physiological role for TLE proteins may be to prevent the expression of AML responsive genes in cells where these genes are not normally expressed. To test this possibility, we monitored the effects of co-expressing AML1, AML2 or AML3 with either TLE1 or TLE2, on AML dependent induction of OC gene transcription in osseous and non-osseous cells. We performed our assays with a reporter gene construct containing 1.1 kb of osteocalcin promoter sequences. The results show that induction of OC promoter activity by AML1, AML2 or AML3 in each case is repressed by co-expression of TLE1 or TLE2 (Fig. 3A). Consistent with the interaction of TLE proteins with the C-terminus of AML transcription factors, no repression was observed when the C terminus of AML1 (Fig. 3B) or AML3 (Fig. 3C) was deleted. In addition, we note that deletion of the C terminus reduces AML-3-dependent activation from 8 to 9-fold to 4 to 5-fold. This result suggests that the C-terminal segment of AML-3 (aa 501 to 513) contributes to both activation and TLE-dependent suppression. The molecular basis for this observation remains to be established. Based on the results presented in Fig. 3, we conclude that TLE proteins are capable of repressing AML dependent activation within the context of the natural bone-specific OC promoter.

Maximal levels of the Groucho/TLE/R-esp protein correlate with minimal expression of the bone-related osteocalcin gene during osteoblast differentiation

Osteocalcin gene expression is repressed and activated during osteoblast differentiation. To further evaluate a role for TLE proteins in AML-dependent gene regulation in osteoblasts, we evaluated TLE expression during osteoblast differentiation. Northern and western blot analyses of rat calvarial osteoblasts show that TLE mRNA and protein are expressed at all

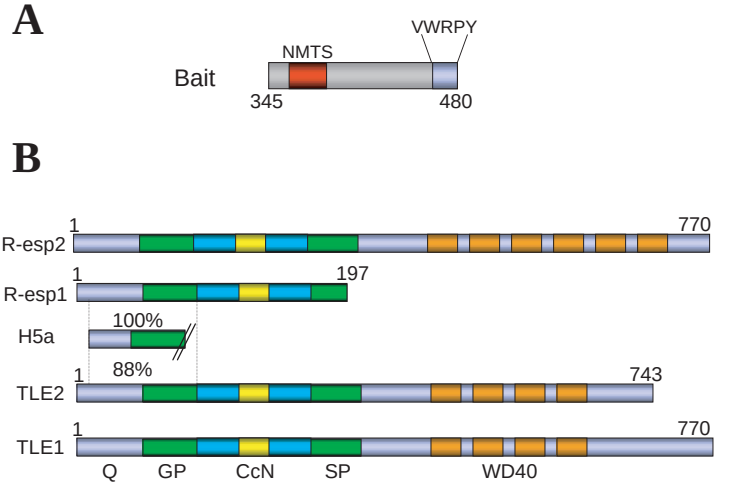

Fig. 2. TLE proteins interact with the C terminus of AML1 in a yeast two-hybrid assay: (A) Diagram of the AML1 fragment (345-480) used as bait. Yeast colonies containing the bait alone or containing the H5a insert did not grow on plates with the appropriate selective medium (see Materials and Methods). X-gal assays confirmed the positive interaction. (B) The initial DNA sequence of the X-gal positive clone (H5a) isolated by the yeast two hybrid screening shows 100% identity with the rat R-esp1, R-esp2 and 88% identity with human TLE1 and TLE2. The H5a clone extend beyond the segment shown, but the exact 3′ end has not been established (double diagonal lines). Distinct domains in each protein are indicated with different colored boxes: Q, glutamine-rich domain; GP, glycine and proline rich domain; CcN, domain containing the nuclear localization sequence; SP, serine and proline rich domain; WD, Tandem repeats of tryptophan and aspartate.

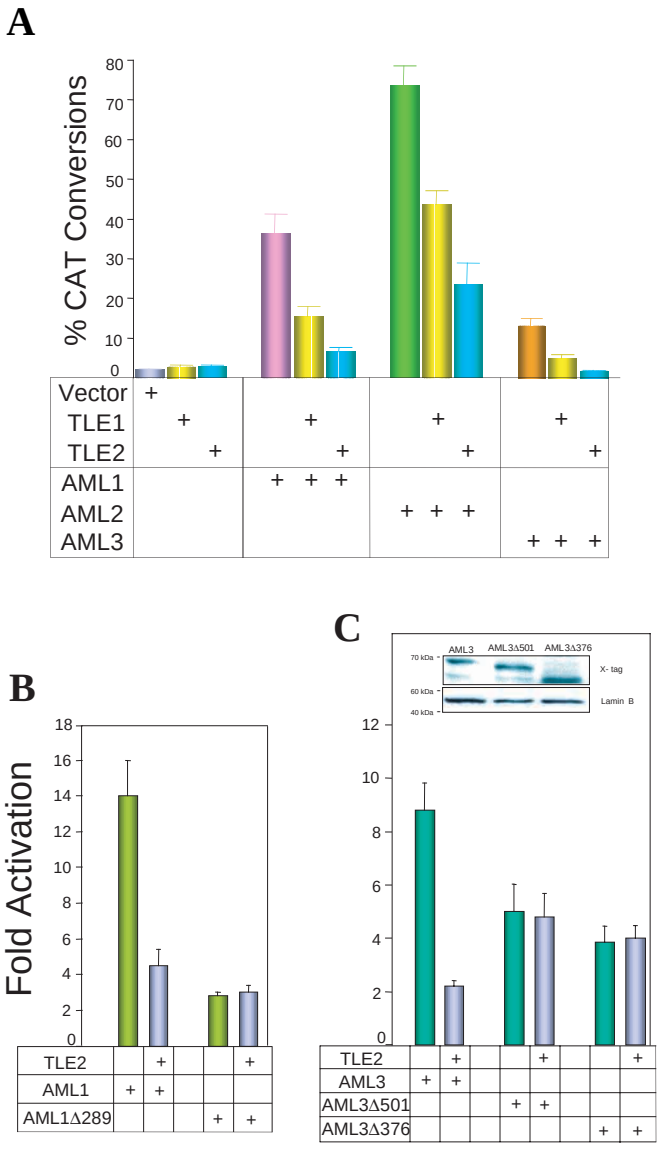

Fig. 3. TLE protein downregulates AML mediated activation of the OC gene through interaction with the VWRPY motif: (A) TLE represses AML activation of the osteocalcin promoter. HeLa cells were transiently co-transfected with either 0.75 μg of CMV empty vector, TLE and/or AML expression constructs (as indicated) and 2.5 μg of 1.1 kb OC promoter-CAT reporter. Reporter activities were assayed 24-36 hours after transfection. CAT activity was normalized for luciferase values, used as internal control and the data presented as % CAT conversions. (B and C) C terminus and VWRPY-dependent repression of AML1 (B) and AML3 (C) mediated activation of the osteocalcin gene promoter by TLE2. HeLa cells were co-transfected with –1.1 kb OC-CAT, TLE2 and AML1 or C terminus deletion (289) constructs as indicated (B). The expression and stability of truncated proteins are comparable to the full length protein (Zeng et al., 1997). HeLa cells were co-transfected with –1.1 kb OC-CAT, TLE2 and AML-3 full length, C terminus deletion (Δ376) or VWRPY deletion mutant (Δ501) constructs (C). The insert shows by western blot analyses that the expression and stability of truncated AML-3 proteins are comparable to the full length protein. The %CAT conversion was normalized to luciferase values and the bar graph shows the fold activation (expression construct/empty vector). Each bar represents the LS + s.e.m. (n=15), P<0.01.

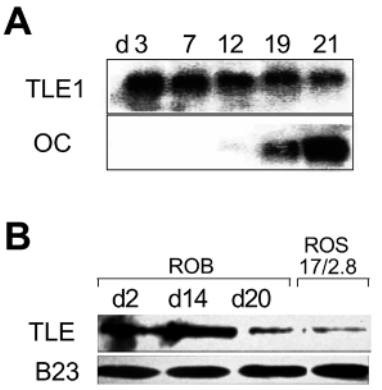

Fig. 4. Reciprocal expression of TLE and osteocalcin during osteoblast differentiation. (A) Northern blot analyses demonstrate the expression pattern of TLE1 during in vitro rat osteoblast differentiation. Total cellular RNA was isolated from cells at different stages of differentiation: day 3-7 (proliferation), day 12 (matrix maturation), and day 19-21 (mineralization). The total RNA (20 μg/lane) was then resolved on 1% agarose gel, transferred to a nylon membrane and hybridized with a TLE1 or rat osteocalcin (OC) probe. (B) Western blot analyses of whole cell proteins extracted from the rat calvaria osteoblast cultures at different time points (d2, 14 and 20) represent growth and differentiation and ROS 17/2.8 osteosarcoma cells at confluence. A total of 30 μg protein/lane was resolved in a 12% SDS-PAGE and blotted with anti-TLE antibody. A 95 kDa band corresponding to the TLE protein(s) was detected. The nucleolin B23 antigen is shown as an internal control for protein loading.

Table 1. Summary of the in situ association of AML and TLE proteins in osseous and non-osseous cells

AML/CBFα PEBP2/RUNX	TLE/ Groucho/ R-esp	% Foci colocalized			
		HeLa		ROS 17/2.8	
		WC	NMIF	WC	NMIF
AML1	TLE1	60	60	64	59
	TLE2	48	52	51	37
AML2	TLE1	64	63	61	63
	TLE2	55	55	50	46
AML3	TLE1	66	66	61	55
	TLE2	59	53	53	54
AML3 Δ376	TLE1	3	–	5	–
	TLE2	3	–	3	–

Representative HeLa and ROS 17/2.8 cells expressing TLE and AML proteins were sectioned with 0.25 μm spacing in Z plane. The number of foci containing both AML and TLE proteins were calculated for each section. Pooled data from five randomly selected cells (13-20 sections per cell) are presented as '% colocalization'. The standard deviation is less than ±2.1 for each group.

developmental stages examined (i.e. d2, proliferation stage; d14, post-proliferative transition; and d21, differentiated stage; Fig. 4). Interestingly, the levels of TLE are downregulated between d14 and d20/d21 which coincides with the induction of OC gene expression in mature osteoblasts. Hence, the abundance of TLE proteins in immature osteoblasts may attenuate OC gene expression, and downregulation of TLE protein levels in differentiated osteoblasts may facilitate maximal expression of the OC gene.

In situ localization of the Groucho/TLE proteins with AML transcription factors and association with the nuclear matrix

Functional interactions between TLE and AML1 proteins have been defined by genetic and/or biochemical assays, but there is limited insight into the association of AMLs and TLE proteins within the cell. We performed digital immunofluorescence and confocal microscopy using whole cell and nuclear matrix preparations from both osseous and non-osseous cells transiently co-transfected with TLE and AML expression constructs (see Figs 7, 8, 9; Table 1; data not shown). Because AML proteins are components of the nuclear matrix (Merriman et al., 1995; Zeng et al., 1997), we assessed whether TLE proteins are also targeted to this subnuclear compartment.

The microscopy results using whole cell preparations show that the Xpress tagged TLE1 (Fig. 5) and TLE2 (Fig. 6) exhibit a punctate distribution throughout the nucleus but are excluded from the nucleoli. Sequential detergent and salt extractions of cells (NMIF preparation) selectively remove soluble components of the cytoplasmic compartment, as well as soluble nuclear and chromatin-related proteins. The punctate pattern of TLE proteins that is observed in whole cells is retained in the nuclear matrix-intermediate filament preparations. The subnuclear distribution and relative strength of the immunofluorescence signals are comparable for whole cell and NMIF preparations. Consistent with previous observations (Stifani et al., 1992), we detect a pool of soluble TLE2 in the cytoplasmic compartment in both HeLa (Fig. 6) and ROS 17/2.8 cells (data not shown), whereas TLE1 (Fig. 5) appears to be restricted to the nucleus. Taken together, our data indicate that TLE1 and TLE2, similar to AML transcription factors, are associated with the nuclear matrix and suggest that TLE-dependent repression of AML responsive genes may involve architectural components of the nucleus.

To assess directly whether subnuclear foci containing TLE proteins also associate with AML factors, we performed double-label immunofluorescence microscopy of HeLa or ROS 17/2.8 cells transiently transfected with constructs encoding TLE and AML proteins (Figs 7-10; Table 1). The results show that a significant fraction (40-70%) of AML1 (Fig. 7), AML2 (Fig. 8) and AML3 (Fig. 9) foci each associate with TLE1 or TLE2 in both whole cell and NMIF preparations of HeLa cells. Similar results were obtained for ROS 17/2.8 cells (Table 1). In contrast,

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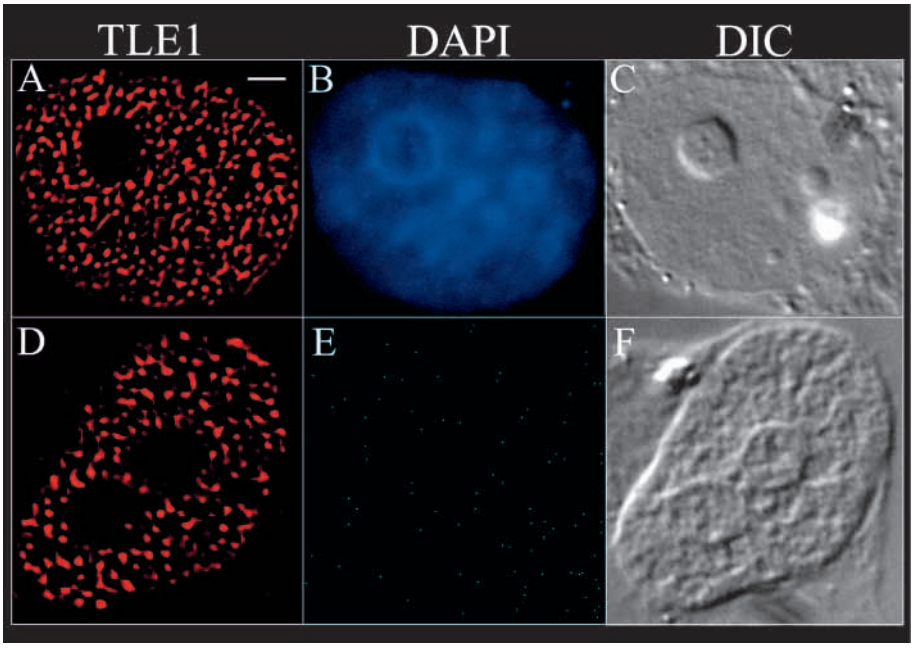

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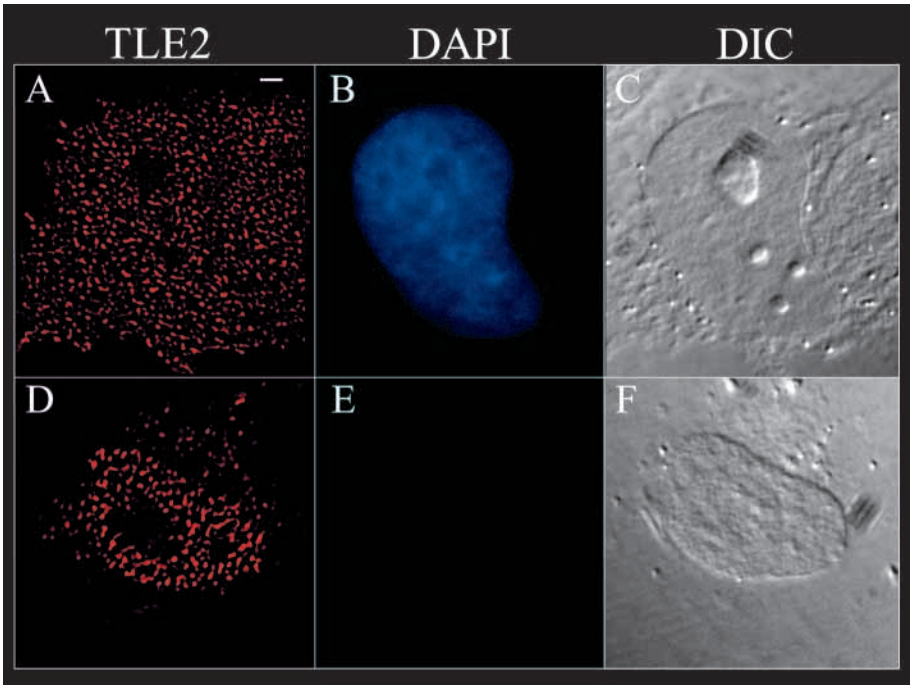

Figs 5 and 6. TLE1 and TLE2 are nuclear matrix proteins. TLE1 (Fig. 5) or TLE2 (Fig. 6) expression constructs (2 μg) were transiently transfected into HeLa cell grown on coverslips. Cells were extracted for in situ nuclear matrix preparation (see Materials and Methods). Whole cell (A-C) or nuclear matrix preparations (D-F) are shown. Xpress tag TLE1 or TLE2 (red signal) were detected with a mouse monoclonal anti-Xpress antibody and Alexa 568 goat anti-mouse secondary antibody. DAPI-detection of DNA in whole cells and absence in NMIF; DIC, differential interference contrasts. Bar, 2 μm.

a truncated AML protein lacking the C terminal region (AML3Δ376) does not show significant co-localization with TLE proteins (Fig. 10; Table 1). The absence of AML3Δ376 and TLE co-localization is in agreement with the properties of this truncated protein which: (i) lacks the TLE/Groucho interacting domains, (ii) does not support TLE/Groucho repression of AML-dependent activation, and (iii) does not contain a nuclear matrix targeting signal that supports subnuclear targeting to AML foci involved in C terminus-dependent gene regulation.

DISCUSSION

In this study, we show that four natural AML dependent promoters (i.e. OC, MDR, LTR and BSP) are either activated or repressed by AML proteins. Our data suggest that repression involve at least two distinct segments within the C terminus of AML proteins, and that promoter context dependent repression (or activation) is a shared property of AML1, AML2 and AML3. We note that our findings are not related to variations in expression levels of WT and mutant AML factors as shown by Zeng et al. (1997) and Tang et al. (1999) and western analyses in these studies (Figs 1 and 3). Thus, AML factors may at least in part functionally compensate for each other and have shared biochemical properties. Using yeast two hybrid assays and mammalian co-expression analyses, we provide additional support for the emerging concept that the C terminus of AML1 interacts with a repressor protein of the Groucho/TLE/R-esp class to repress AML mediated transcriptional activation in a promoter dependent manner. Using digital immunofluorescence and confocal microscopy, we show that Groucho proteins TLE1 and TLE2 like AML are associated with the nuclear matrix and that a significant subset of TLE foci colocalized with AML proteins. Furthermore, our data suggest that TLE repression may be physiologically relevant to spatio-temporal activation of AML dependent tissue specific genes.

Our yeast two hybrid analysis using the AML1 C terminus has resulted in the detection of a protein that has previously been implicated in AML dependent transcriptional control. Thus, our unbiased screen for AML1 partner proteins, which was performed with a unique bait construct and a bone-related cDNA library, corroborates other studies employing distinct experimental approaches which have indicated that TLE proteins regulate AML-dependent transcription (Aronson et al., 1997; Imai et al., 1998; Levanon et al., 1998; Thirunavukkarasu et al., 1998).

We have previously shown that AML factors reside in subnuclear domains by a specific intranuclear targeting signal (McNeil et al., 1999; Merriman et al., 1995; Tang et al., 1999; Zeng et al., 1997). The nuclear matrix may support transcriptional regulation by concentrating and localizing gene regulatory factors, as well as by facilitating the formation and maintenance of transcriptionally active chromatin (Guo et al., 1995; Javed et al., 1999; Stein et al., 1998; Zeng et al., 1998). Our observation that both AML and TLE proteins are

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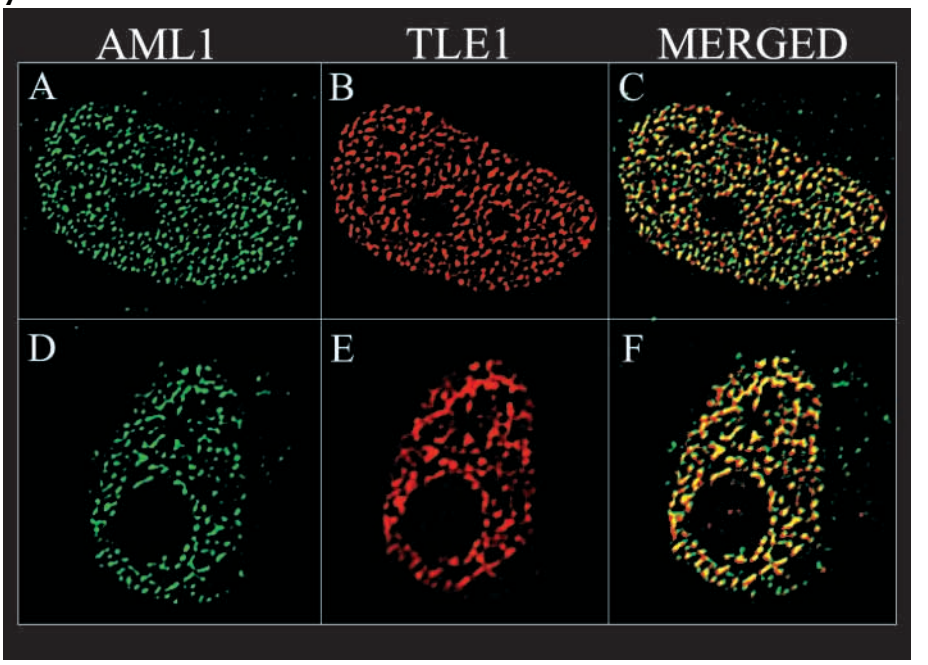

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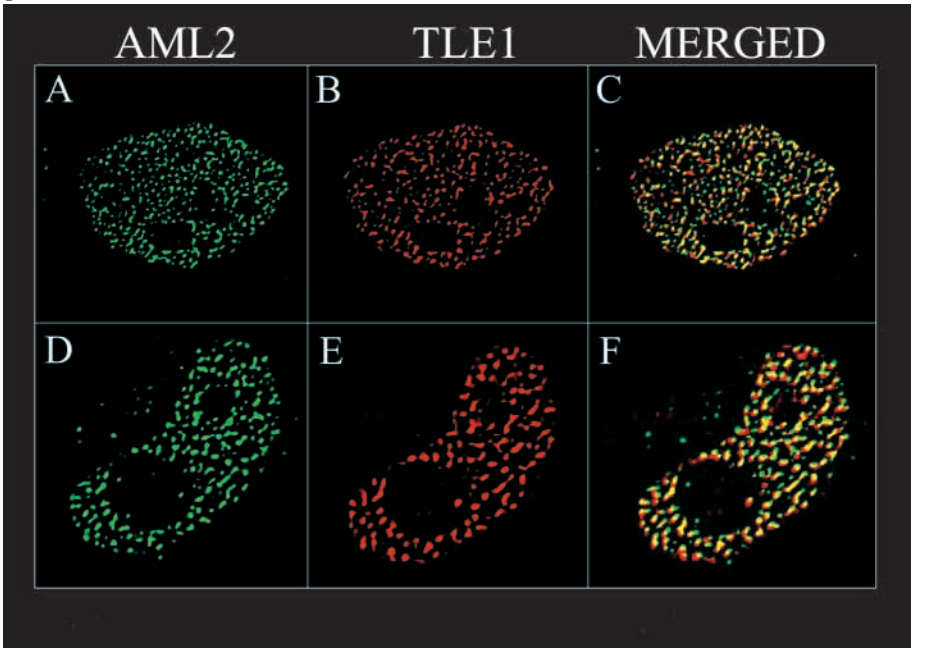

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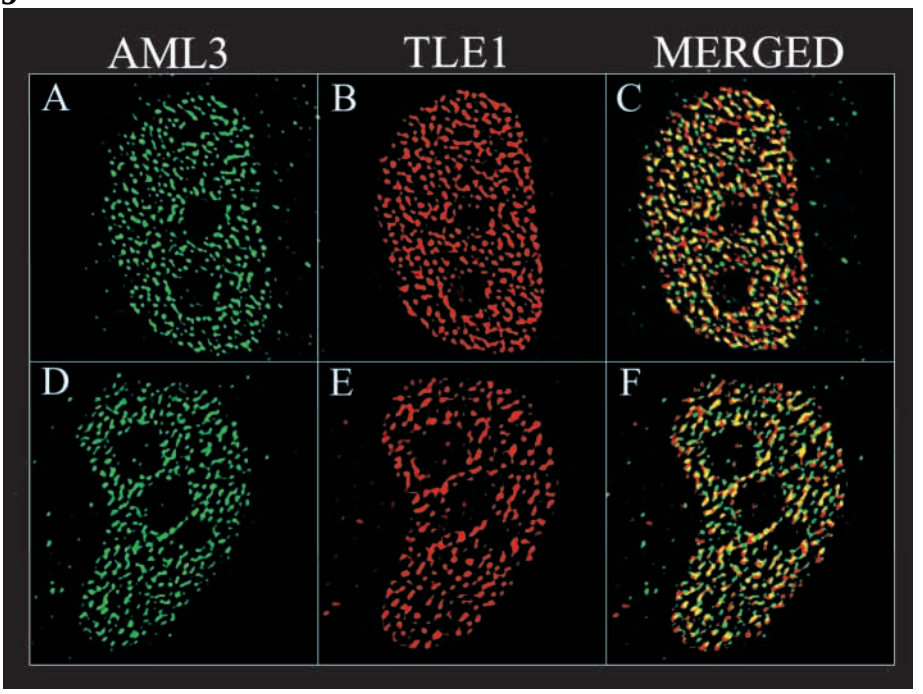

Figs 7, 8 and 9. Double label immunofluorescence analysis of TLE and AML factors at the nuclear matrix in situ: AML1 (Fig. 7), AML2 (Fig. 8) and AML3 (Fig. 7) were co-transfected with TLE1 or TLE2 into HeLa cells and processed for in situ nuclear matrix preparations. Cell preparations were double stained for AML and TLE proteins (see Materials and Methods). Images of cell sections were acquired at 0.25 μm intervals in the Z plane and deconvolved after determining the microscope-camera point spread function. Representative images for each AML transcription factor with TLE1 are shown as whole cell (A-C) and nuclear matrix (D-F) preparations.

associated with the nuclear matrix suggests that one component of the mechanism of TLE repression may involve formation of stable macro-molecular complexes with architectural components of the nucleus.

Our data indicate that association of TLE with the nuclear matrix is cell type independent, and we observe a similar pattern of association in osseous and non-osseous cells. We also show that association of TLE1 or TLE2 with the nuclear matrix does not depend upon interaction with AML proteins, as we observe TLE nuclear matrix association in HeLa cells that lack AML factors. However, we find that co-localization of TLE with AML is dependent on interaction with the C terminus of AML.

Recent data from our laboratory indicate that AML factors may regulate tissue-specific gene expression by facilitating chromatin remodeling and/or modification in nucleosomal organization (Javed et al., 1999). Several other observations are consistent with chromatin related mechanisms mediating AML dependent transcriptional control. For example, AML factors are known to interact with the co-repressor protein Sin3A (Lutterbach et al., 1998a). Similarly, Groucho/TLE proteins have been shown to associate with histone H3, and histone deacetylase Rpd3 (Chen et al., 1999; Palaparti et al., 1997). Hence, developmental changes in TLE repression of AML activation may result from temporal modifications in the chromatin-related interactions among AML, TLE, histone H3 and/or component of the nuclear architecture.

Transcription of the osteocalcin gene is stringently regulated during

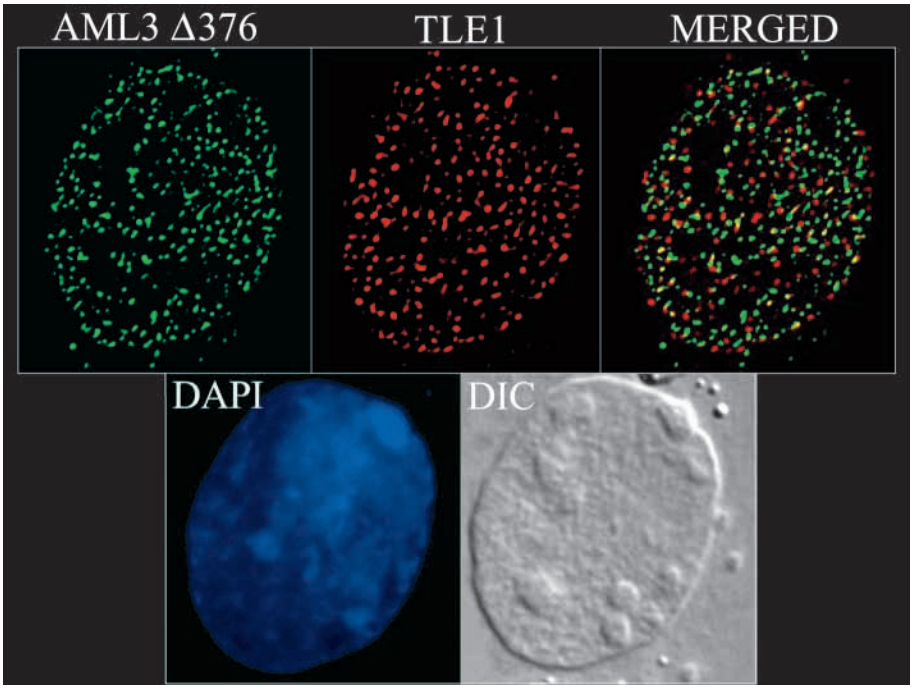

Fig. 10. Deletion of C terminus of AML3 abolishes co-localization with TLE proteins. AML3 deletion mutant (Δ376) was co-transfected with TLE1 or TLE2 in HeLa cells. Shown are immuno- positive whole cells and the corresponding DAPI signals (to reveal nuclei), as well as differential interference contrast (DIC) images. Image restoration and deconvolution were performed as described in Materials and Methods.

osteoblast differentiation (Stein and Lian, 1993) and OC gene induction is coupled to a post-proliferative increase in AML3 DNA binding activity (Banerjee et al., 1997; Ducy et al., 1997). However, AML proteins are present in proliferative osteoblasts prior to the onset of OC gene expression. The ability of Groucho-class proteins to repress AML dependent activation may have a physiological function by maintaining AML responsive genes repressed until other rate-limiting developmental cues support the stage-specific induction of OC gene expression in osteoblasts. In a broader context, the activities of AML proteins and transcriptional activation of AML responsive genes may be regulated in part during skeletal development and hematopoiesis by controlling the level of TLE proteins.

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