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Intranuclear Trafficking of RUNX/AML/CBFA/PEBP2 Transcription Factors in Living Cells: A Dissertation

Kimberly Stacy Harrington

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Intranuclear Trafficking of RUNX/AML/CBFA/PEBP2 Transcription Factors in Living Cells

by

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Intranuclear Trafficking of RUNX/AML/CBFA/PEBP2 Transcription Factors in Living Cells

A Dissertation Presented

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Department of Cell Biology

March 28, 2003
Dedication

To My Parents,
Gail and Joseph Harrington
ACKNOWLEDGMENTS

Foremost, I thank my loving parents, Gail and Joseph Harrington, for their paramount of support throughout my life both in and out of school. I am grateful for my sister, Lori, and brothers, Joe and Jason, for their encouragement through the years to excel and obtain my Ph.D. even though I will never hear the end that I was the first to go to graduate school and the last to graduate.

I show extreme gratitude to my thesis mentor, Janet Stein, as well as Gary Stein, Jane Lian and Andre van Wijnen for their advice and support throughout my graduate career. I show great appreciation to Yu-Li Wang for his assistance with the FRAP analysis experiments.

I show immense admiration to my best friend, Hicham Drissi, whose support and friendship made work and life enjoyable everyday.

I thank my friends, fellow graduate students and co-workers, Faiza Afzal, Mai Luong, Caroline van der Meijden, Daron Forman, Nicole Faulkner, Stan Stachelek, Hidenori Meiseki, Inge Smoorenburg, John Ribadeneyra, Humberto Rossi, Amjad Javed, Soraya Gutierrez, Kaleem Zaidi, Rong-Lin Xie, Beata Paluch, Brian Cho, Liz Buffone, Sandra McNeil, Bonnie Bidinger, Pat-Sue Vaughn, Chris Lengner, Heather Steinman, Ricardo Medina, Angela Miele, Jitesh Pratap, Jiali Shen, Ada Staal, Arlyssa LaPorte, Christian Koolloos, Rahul Tare, Dan Young and Brune Trevant for making work in the Stein/Lian lab and life outside of the lab entertaining, pleasurable and a great experience. I would like to give a special thanks to Rosa Mastrodatro and Shirwin Pockwise for all their hard work keeping the lab running efficiently.
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The family of runt related transcription factors (RUNX/Cbfa/AML/PEBP2) are essential for cellular differentiation and fetal development. RUNX factors are distributed throughout the nucleus in punctate foci that are associated with the nuclear matrix/scaffold and generally correspond with sites of active transcription. Truncations of RUNX proteins that eliminate the C-terminus including a 31-amino acid segment designated the nuclear matrix targeting signal (NMTS) lose nuclear matrix association and result in lethal hematopoietic (RUNX1) and skeletal (RUNX2) phenotypes in mice. These findings suggest that the targeting of RUNX factors to subnuclear foci may mediate the formation of multimeric regulatory complexes and contribute to transcriptional control. In this study, we hypothesized that RUNX transcription factors may dynamically move through the nucleus and associate with subnuclear domains in a C-terminal dependent mechanism to regulate transcription. Therefore, we investigated the subnuclear distribution and mobility of RUNX transcription factors in living cells using enhanced green fluorescent protein (EGFP) fused to RUNX proteins. The RUNX C-terminus was demonstrated to be necessary for the dynamic association of RUNX with stable subnuclear domains. Time-lapse fluorescence microscopy showed that RUNX1 and RUNX2 localize to punctate foci that remain stationary in the nuclear space in living cells. By measuring fluorescence recovery after photobleaching, both RUNX1 and RUNX2 were found to dynamically and rapidly associate with these subnuclear foci with a half-time of recovery in the ten-second time scale. A large immobile fraction of RUNX1 and RUNX2 proteins
was observed in the photobleaching experiments, which suggests that this fraction of RUNX1 and RUNX2 proteins are immobilized through the C-terminal domain by interacting with the nuclear architecture. Truncation of the C-terminus of RUNX2, which removes the NMTS as well as several co-regulatory protein interaction domains, increases the mobility of RUNX2 by at least an order of magnitude, resulting in a half-time of recovery equivalent to that of EGFP alone.

Contributions of the NMTS sequence to the subnuclear distribution and mobility of RUNX2 were further assessed by creating point mutations in the NMTS of RUNX2 fused to EGFP. The results show that these point mutations decrease, but do not abolish, association with the nuclear matrix compared to wild-type EGFP-RUNX2. Three patterns of subnuclear distribution were similarly observed in living cells for both NMTS mutants and wild-type RUNX2. Furthermore, the NMTS mutations showed no measurable effect on the mobility of RUNX2. However, the mobility of RUNX proteins in each of the different subnuclear distributions observed in living cells were significantly different from each other. The punctate distribution appears to correlate with higher fluorescence intensity, suggesting that the protein concentration in the cell may have an effect on the formation or size of the foci. These findings suggest that the entire NMTS and/or the co-regulatory protein interaction domains may be necessary to immobilize RUNX2 proteins.

Because RUNX factors contain a conserved intranuclear targeting signal, we examined whether RUNX1 and RUNX2 are targeted to common subnuclear domains. The results show that RUNX1 and RUNX2 colocalized in common subnuclear foci. Furthermore, RUNX subnuclear foci contain the co-regulatory protein CBFβ, which
heterodimerizes with RUNX factors, and nascent transcripts as shown by BrUTP incorporation. These results suggest that RUNX subnuclear foci may represent sites of transcription containing multi-subunit transcription factor complexes.

RUNX2 transcription factors induce expression of the osteocalcin promoter during osteoblast differentiation and to study both RUNX2 and osteocalcin function, it would be helpful to have transgenic mice in which OC expression could be easily evaluated. Therefore, to assess the in vivo regulation of osteocalcin by RUNX protein, we generated transgenic mice expressing EGFP controlled by the osteocalcin promoter. Our results show that EGFP is expressed from the OC promoter in a cultured osteosarcoma cell line, but not in a kidney cell line, and is induced by vitamin D3. Furthermore, the OC-EGFP transgenic mice specifically express EGFP in osteoblasts and osteocytes in bone tissues. Moreover, EGFP is expressed in mineralized bone nodules of differentiated bone marrow derived from transgenic mice. Thus, these mice produce a good model for studying the in vivo effects of RUNX-mediated osteocalcin regulation and for developing potential drug therapies for bone diseases.

Taken together, our results in living cells support the conclusion that RUNX transcription factors dynamically associate with stationary subnuclear foci in a C-terminal dependent mechanism to regulate gene expression. Moreover, RUNX subnuclear foci represent transcription sites containing nascent transcripts and co-regulatory interacting proteins. These conclusions provide a mechanism for how RUNX transcription factors may associate with subnuclear foci to regulate gene expression. Furthermore, the OC-EGFP transgenic mice now provide a useful tool
for studying the in vivo function and regulation of osteocalcin by RUNX proteins during osteoblast differentiation and possibly for developing therapeutic drugs for treatment of bone diseases in the future.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>CBF</td>
<td>Core binding factor</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CSK</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase I</td>
<td>Deoxyribonuclease I</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyl transferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HES-1</td>
<td>Hairy and Enhancer of Split 1</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NMIF</td>
<td>Nuclear matrix-intermediate filament</td>
</tr>
<tr>
<td>NMTS</td>
<td>Nuclear matrix targeting signal</td>
</tr>
<tr>
<td>OB</td>
<td>Osteoblast</td>
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<tr>
<td>OC</td>
<td>Osteocalcin</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEBP2</td>
<td>Polynoma enhancer binding protein 2</td>
</tr>
<tr>
<td>RHD</td>
<td>Runt homology domain</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Rat osteosarcoma cells</td>
</tr>
<tr>
<td>RUNX</td>
<td>Runt-related transcription factor X</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SaOS-2</td>
<td>Human osteosarcoma cells</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>Switching mating type (SWI)/ Sucrose nonfermenting (SNF)</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D response element</td>
</tr>
<tr>
<td>WC</td>
<td>Whole cell</td>
</tr>
<tr>
<td>YY1</td>
<td>Yin yang 1</td>
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The Nucleus: Organizing Center for Transcriptional Regulation

The nucleus has several biological functions including gene expression, DNA replication and DNA repair. The nucleus is compartmentalized and factors involved in several of these cellular functions are localized to specific punctate subnuclear domains (Nickerson et al., 1995; Stein et al., 2000b; Lamond and Earnshaw, 1998; Cook, 1999; Berezney et al., 2000; Misteli and Spector, 1998; Davie et al., 1997). Transcription is a necessary cellular process that synthesizes mRNA in the nucleus. Sites of active transcription are visualized by labeling nascent transcripts with 5-bromouridine 5'-triphosphate (BrUTP) (Wansink et al., 1993). Transcription requires the assembly of co-regulatory proteins and the RNA polymerase holoenzyme on the promoter region of a specific gene (Strouboulis and Wolffe, 1996; Näär et al., 2001). To activate transcription, a conformational change in chromatin structure is required for the assembly of transcription factor machinery.

Chromatin is organized in chromosomal territories in the nucleus. Transcription and splicing occur in the functional compartment called the perichromatin fibrils, which are found at the boundaries of condensed chromatin domains. Active genes are positioned in euchromatin located in the interior of the nucleus and inactive genes are localized to condensed heterochromatin on the periphery of the nucleus (Strouboulis and Wolffe, 1996; Jackson et al., 1997; Näär et al., 2001). Inactive chromatin is tightly packaged into higher order 30 nm chromatin fibers (Figure 1). Active chromatin opens into 10 nm chromatin fibers and is sensitive to nuclease digestion as shown by the appearance of nuclease hypersensitive sites (Jackson, 1997; Davie et al., 1997; Näär et al., 2001). Chromatin fibers are
Figure 1. Nuclear Organization and Structure. Inactive chromatin is organized into highly condensed 30 nm fibers. Active chromatin is organized into chromatin loops, which attach to the nuclear matrix (purple filaments, shown below) at matrix attachment regions (MAR). Histone acetylation and chromatin remodeling factors associate with chromatin to open active chromatin into 10 nm fibers, which have the appearance of ‘beads-on-a-string’. The 10 nm fibers are composed of 200 bp of DNA wrapped around a histone octamer. The histone octamer consists of two copies each of the histones H2A, H2B, H3 and H4. Histone H1 associates with the DNA linker sequence. Promoters in an open conformation contain nucleotide sequences that are recognized by transcription factors. Histone acetylation and chromatin remodeling factors disassociate nucleosomes from active promoters, so transcription factors can bind to DNA and regulate gene expression (shown in image below). (Adapted from Stein et al. (2003). J. Cell. Biochem. 88, 340-355.)
5'ACCACA 3'
organized into loop domains with matrix attachment regions (MARs) attaching to the nuclear matrix at the base of the loops (Davie, 1997; Stein et al., 2003; Galande and Kohwi-Shigematsu, 2000). Transcriptionally active genes are localized to decondensed chromatin loops (Figure 1; Davie, 1997; Stein et al., 2003). The 10 nm chromatin fibers have the appearance of ‘beads-on-a-string’ which are composed of ~200 bp DNA segments tightly wrapped around a histone octamer to form a nucleosome. The nucleosome octamer consists of a histone (H3-H4)_2 tetramer surrounded on either side by two H2A-H2B dimers (Figure 1). Histone H1 binds to the linker DNA that groups the nucleosomes together and stabilizes the chromatin into the 30 nm fibers (Davie, 1997; Nääär et al., 2001; Jackson et al., 1997).

Transcriptional activation requires the opening of condensed chromatin, which is the result of acetylation of the N-terminal tails of the core histones (Davie, 1997; Nääär et al., 2001). The level of acetylation is related to the balance between the activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). In general, transcriptionally active chromatin has hyperacetylated histones, whereas inactive chromatin is hypoacetylated. Acetylation of histones weakens the nucleosome interaction with the DNA, allowing for chromatin to open so transcription factors can bind (Davie, 1997; Nääär et al., 2001). Furthermore, chromatin remodeling factors, such as SWI/SNF complexes open chromatin by disassociating DNA from nucleosomes or by sliding nucleosomes along the DNA in an ATP-dependent mechanism to make gene promoters accessible to the transcription machinery (Morales et al., 2001; Nääär et al., 2001). The order in which histone acetyltransferases, chromatin remodeling factors and the transcription machinery
associate with gene promoters is specific for each promoter (Narlikar et al., 2002). One family of transcription factors, RUNX, is the focus of our study and has been shown to interact with a histone acetyltransferase, p300/CBP, to activate transcription and a histone deacetylase, HDAC6, to repress transcription (Kibatayshi et al., 1998; Sierra et al., 2003; Westendorf et al., 2002).

**Trafficking of Proteins by Nuclear Transport**

Nucleo-cytoplasmic transport of proteins and RNA occurs through nuclear pore complexes (NPC), which connect the cytoplasm to the nucleus and are located in the nuclear envelope (Wente, 2000). The NPC is formed from a family of proteins called nucleoporins. Small molecules (<40 kDa) passively diffuse through the NPCs (Ossareh-Nazari et al., 2001). Larger proteins and RNA molecules move through the NPCs through controlled mechanisms. Larger nuclear proteins can enter the nucleus through the presence of a simple or bipartite basic amino acid sequence called the nuclear localization sequence (NLS), which actively transports the molecule against the concentration gradient. Import of nuclear proteins (cargo) requires the NLS to bind to an adaptor protein (karyopherins/ importins α) that interacts with a transport receptor (karyopherins/ importins β). The cargo-karyopherin complex then interacts with the nucleoporins in the NPC, associates with small guanosine triphosphatase Ran (RanGTP) protein and is transported to the nucleus (Wente, 2000).

Nuclear export involves the presence of a nuclear export signal (NES), which was originally discovered in the human immunodeficiency virus (HIV)-1 Rev protein and is a 10-amino acid hydrophobic leucine-rich sequence (Ossareh-Nazari et al.,
NESSs are present in several cellular and viral proteins involved in transcription, cell cycle and signal transduction. The export of proteins from the nucleus requires the export receptors (termed exportins), which have a similar sequence and function to importins and belong to the same karyopherin superfamily. RUNX transcription factors are nuclear proteins, which are localized to the nucleus through a NLS.

**RUNX Family of Transcription Factors Regulate Tissue-Specific Gene Expression**

RUNX transcription factors belong to the Runt homology family of proteins, which is composed of at least two members in *Drosophila melanogaster* namely *runt* and *lozenge*. There are three mammalian RUNX family members encoded by independent genes: RUNX1/AML1/Cbfa2/PEBP2αB, RUNX2/AML3/Cbfa1/PEBP2αA and RUNX3/AML2/Cbfa3/PEBP2αC (Figure 2; Levanon et al., 1994). Gene disruptions in mice show that each of the RUNX proteins has a tissue-specific phenotype (Komori et al., 1997; Otto et al., 1997; Wang et al., 1996a; Okuda et al., 1996; North et al., 1999; Li et al., 2002; Levanon et al., 2002). RUNX proteins share several functionally conserved domains including the Runt Homology Domain (RHD), the nuclear localization signal (NLS) and a subnuclear localization signal called the nuclear matrix targeting signal (NMTS) (Speck and Stacy, 1995; Zeng et al., 1997; Zaidi et al., 2001). RUNX factors share 66% homology with *Drosophila runt* protein and over 90% homology with each other within the 128-amino acid RHD (Speck and Stacy, 1995). RUNX proteins are shown
Figure 2. Structure and Nomenclature of RUNX/CBF/AML/PEBP2 Proteins.

(A) There are three RUNX α-subunit proteins, RUNX1, RUNX2 and RUNX3. The three RUNX proteins contain conserved amino acid sequences in the following functional domains: 1) the runt homology domain (RHD); 2) nuclear localization signal (NLS); 3) nuclear matrix targeting signal (NMTS); and 4) the repression domain (VWRPY). RUNX2 has a unique domain of a glutamine-alanine amino acid stretch (QA). The AML-1B isoform of the RUNX1 protein is 480 amino acids; the PEBP2αA isoform of RUNX2 is 513 amino acids and the p44 isoform of RUNX2 is 415 amino acids. (B) The nomenclature for the RUNX factors produced three names that were given from different laboratories for the same protein. RUNX was the agreed upon nomenclature by the Human Genome Organization. AML was designated for its involvement in Acute Myeloid Leukemia (Levanon et al., 1994). CBF was designated as Core Binding Factor for its ability to bind to the core binding sequence in the Moloney murine leukemia virus enhancer and the SL3-3 murine leukemia virus enhancer (Speck and Stacy, 1995). PEPB2 was designated as Polyoma Enhancer Binding Protein 2 for its association with the polyoma virus enhancer A (Bae et al., 1994). Human chromosomes on which the RUNX and CBFβ genes are located are shown in the last column.
to function in a heterodimeric complex that contains an α-subunit and a β-subunit. The α-subunit or RUNX recognizes and binds to the DNA consensus sequence Pu/TACCPuCA (Meyers et al., 1993; Speck and Stacy, 1995). This core sequence is present in a variety of gene promoters that control cellular differentiation. The RHD is responsible for RUNX interaction with DNA and heterodimerization with the β-subunit (CBFβ) (Meyers et al., 1993; Speck and Stacy, 1995). CBFβ does not bind directly to DNA yet its interaction with RUNX proteins enhances DNA binding affinity of RUNX (Speck and Stacy, 1995; Wang et al., 1993; Ogawa et al., 1993a; Harada et al., 1999).

Each RUNX family member has several isoforms resulting from alternative splicing and usage of different promoters, a distal promoter (P1) and a proximal promoter (P2) (Xiao et al., 1999; Fujiwara et al., 1999; Miyoshi et al., 1995; Rini and Calabi, 2001; Bangsow et al., 2001). The RUNX2 gene encodes at least two mRNA isoforms, which produce proteins with different N-termini. One isoform, PEBP2αA (Type I), uses the proximal promoter and produces a 513 amino acid protein starting with the amino acids MRIPV (Ogawa et al., 1993b). The other isoform, til-1 (Type II), uses the distal promoter and generates a 528 amino acid protein with a 15 amino acid extension at its N-terminus compared to Type I and begins with the amino acids MASNS (Stewart et al., 1997). It is possible that the N-terminal extensions have the potential of differentially regulating tissue specific genes. However, it is more likely that these N-termini are simply a vestige of the requirement to regulate RUNX proteins from two different promoters.
RUNX transcription factors function to regulate certain genes required for tissue-specific development (Okuda et al., 1996; Wang et al., 1996a; Komori et al., 1997; Otto et al., 1997; Ducy et al., 1997; Javed et al., 2001; Westendorf and Hiebert, 1999; Ito, 1999; Lutterbach and Hiebert; 2000). The C-termini of RUNX factors have both activation and inhibitory domains that are important for transcriptional regulation of specific genes. The boundaries of the domains responsible for transcriptional regulation are specific depending on the context of the promoter (Westendorf and Hiebert, 1999; Ito, 1999; Lutterbach and Hiebert; 2000). RUNX proteins were originally thought to be transcriptional activators; however they can also function as repressors (Meyers et al., 1995; Javed et al., 2000). RUNX transcription factors regulate many hematopoietic, osteogenic and gastro-intestinal genes involved in growth and differentiation. Among the bone-related genes regulated by RUNX proteins are osteocalcin (OC), osteopontin, bone sialoprotein, collagenase-3, collagen Type I, as well as the RUNX2 gene itself (Harada et al., 1999; Selvamurugan et al., 1998; Ducy et al., 1997; Javed et al., 2001; Cohen, 2001; Lian et al., 2001; Drissi et al., 2000).

Several co-regulatory factors interact with RUNX to form multisubunit transcription regulatory complexes and regulate tissue-specific gene expression (Westendorf and Hiebert, 1999; Ito, 1999). The regions in RUNX1 where several co-regulatory proteins interact are shown in Figure 3. Regulation of specific genes may depend on the co-regulatory proteins that interact with RUNX proteins (Westendorf and Hiebert, 1999; Ito, 1999). Moreover, the spatial and temporal organization of transcription factor multisubunit complexes is important for gene regulation (Stein et
Figure 3. Schematic of the Interacting Regions of Co-regulatory Proteins with RUNX1. A schematic of RUNX1 and the regions of RUNX1 that several co-regulatory proteins interact with are shown. The regions are labeled by a line with the indicated name of the interacting protein under or over the line. The conserved RUNX domains, including the runt homology domain (RHD), the nuclear localization signal (NLS), the nuclear matrix targeting signal (NMST) and the repression domain (VWRPY) are labeled. Several of the co-regulatory proteins, which are illustrated, that have been shown to interact with RUNX1, RUNX2 and/or RUNX3 include Smads (Hanai et al., 1999), Ear-2 (Ahn et al., 1998), ALY (Bruhn et al., 1997), c-Fos and c-Jun (D’Alonzo et al., 2002; Hess et al., 2001), Ets (Erman et al., 1998), C/EBP α, β, and δ (Zhang et al., 1996; Hohaus et al., 1995; Gutierrez et al., 2002), TLE (Imai et al., 1998; Javed et al., 2000; Thirunavukkarasu et al., 1998), Hes1 (McLarren et al., 2001), p300/CBP (Kitabayashi et al., 1998); HDAC6 (Westendorf et al., 2002), mSin3 (Lutterbach et al., 2000), MITF (Ogihara et al., 1999), MEF (Mao et al., 1999), CBFβ (Ogawa et al., 1993a; Wang et al., 1993) and YAP (Yagi et al., 1999).
al., 2000b). For example, a model for the three-dimensional organization of the OC promoter allows for the interaction and cooperation of RUNX with co-regulatory proteins and their association with the nuclear matrix (Stein et al., 1996). Specific combinations of transcription factors assemble onto tissue-specific promoters producing an elaborate network of protein-protein and protein-DNA interactions to regulate gene expression (Ito, 1999). The combination of transcription factors may depend on the gene to be regulated, the specific cell type, and whether the gene will be activated or repressed. For example, the repressor proteins TLE and histone deacetylase can interact with RUNX to inactivate the OC promoter, whereas CBFβ, C/EBP and histone acetyltransferases, such as p300/CBP, can interact with RUNX to activate the OC promoter (Westendorf and Hiebert, 1999; Gutierrez et al., 2002; Javed et al., 2000; Sierra et al., 2003). Thus, the spatial and temporal availability of specific transcription factors and co-factors at a particular place and time in the nucleus may determine which genes are expressed.

Role of RUNX Factors in Development and Disease

The function of RUNX proteins during embryonic development has been studied through gene ablation (Komori et al., 1997; Otto et al., 1997; Wang et al., 1996a; Okuda et al., 1996; North et al., 1999; Li et al., 2002; Levanon et al., 2002). RUNX1 was the first family member to be studied by knockout experiments, which established that RUNX1 is essential for hematopoiesis (Wang et al., 1996a; Okuda et al., 1996). RUNX1 null mice are embryonic lethal and die in utero (E12.5) from hemorrhaging of the central nervous system and failure of definitive hematopoiesis.
Furthermore, mice deficient in the heteromeric protein CBFβ are also embryonic lethal and show a similar spectrum of abnormalities as RUNX1 null mice (Sasaki et al., 1996; Wang et al., 1996b; Niki et al., 1997), indicating that CBFβ is required for biological function of RUNX1. Recently, CBFβ has been shown to be involved in bone development of CBFβ disrupted knock-in mice (Kundu et al., 2002; Yoshida et al., 2002; Miller et al., 2002). Ablation of the RUNX2 gene in mice results in complete lack of bone formation because of a maturational arrest of osteoblast differentiation. RUNX2 null mice die just after birth due to the inability to breathe, and the absence of mineralized skeleton indicates that RUNX2 is required for osteogenesis (Komori et al., 1997; Otto et al., 1997). RUNX3 has recently been shown to be important for the regulation of gastric epithelia and the development of gut and TrkC dorsal root ganglia neurons in RUNX3 knockout mice (Li et al., 2002; Levanon et al., 2002).

The implication of RUNX family members in tissue development is further demonstrated in humans. Mutation of RUNX genes results in a variety of diseases. RUNX1, located on human chromosome 21, is involved in chromosomal translocations that induce acute leukemias. About 40% of human acute leukemias result from translocation of the RUNX1 gene (Westendorf and Hiebert, 1999). The most common RUNX1 chromosomal translocations are t(8;21), t(3;21) and t(12;21), which cause acute myeloid, acute lymphoblastic, and therapy-related leukemias, respectively (Figure 4; Speck and Stacy, 1995; Westendorf and Hiebert, 1999; Ito, 1999). Furthermore, the translocation referred to as inv(16), which involves the fusion of the CBFβ gene (located on human chromosome 16) with the gene for
Figure 4. RUNX1/AML-1 Chromosomal Translocations and the Resulting Fusion Proteins. Chromosomal translocations of RUNX1/AML-1B result in a variety of human acute leukemias. The sites of the translocation on the RUNX1/AML-1B protein are shown as t(8;21), t(3;21) and t(12;21) with arrow to show the amino acid number at the site of translocation. The fusion proteins, which are the result of the translocations are shown below. AML-1/ETO fusion protein is caused by the t(8;21) chromosomal translocation resulting in the fusion of the N-terminal 206 amino acids of AML-1 to amino acid 30 of the eight-twenty-one (ETO) protein. The t(3;21) translocation results in a fusion protein between the first 270 amino acids of AML-1 and the EVI-1 transcription factor. The AML1/ETO and AML1/EVI translocations result in loss of the C-terminal region of AML-1, which contains several co-regulatory protein interaction domains and the NMTS. The t(12;21) translocation results in the fusion of the TEL protein to amino acid 21 of AML-1. This fusion protein retains all of RUNX1 except the N-terminal 21 amino acids. The inv(16) chromosomal translocation, which also results in leukemia is caused by the fusion of the N-terminal 165 amino acids of CBFβ with the myosin heavy chain protein.
smooth muscle myosin heavy chain results in acute myelomonocytic leukemia with eosinophilia (Liu et al., 1993). The levels of RUNX1 proteins also appear to be very important in regulating hematopoiesis as recent studies have shown that RUNX1 haplo-insufficiency induces thrombocytopenia with a predisposition to acute leukemias in humans (Song et al., 1999; Barton and Nucifora, 2000).

Haplo-insufficiency of RUNX2 results in a human autosomal dominant bone disease called cleidocranial dysplasia (CCD). CCD is characterized by patients with open fontanelles and sutures, hypoplastic and aplastic clavicles, short stature and supernumerary teeth (Lee et al., 1997). Several patients with CCD have mutations in the RUNX2 gene, indicating that mutations in RUNX2 cause CCD (Mundlos et al., 1997; Zhang et al., 2000). Overexpression of RUNX2 in mesenchymal cells inhibits osteoblast maturation and causes osteopenia with multiple fractures in mice (Liu et al., 2001), suggesting that the cellular levels of RUNX2 in mesenchymal cells are very important for normal bone development. RUNX3 has been implicated in cancer since loss of RUNX3 expression correlates with the onset of gastric cancer (Li et al., 2002). Taken together, these results show that RUNX proteins are essential for development of hematopoietic, skeletal, neural and gastro-intestinal tissues and that variations in the levels of RUNX expression or disruption of the RUNX genes can lead to serious tissue malformations or cancer.
Nuclear Localization and Nuclear Matrix Association of RUNX Transcription Factors

The nuclear matrix is a nuclear scaffold characterized by electron microscopy as a fibrous network of 10-nm intermediate filaments, and consists of insoluble proteins, nuclear RNAs and <5% DNA (Berezney and Coffey, 1974; Fey et al., 1984, 1986). This internal nuclear matrix scaffold is surrounded by the nuclear envelope consisting of lamins A/C and B and nuclear pore complexes (Stein et al., 2000b). A large network of intermediate filaments connects the internal nuclear matrix to the nuclear envelope surface (Nickerson et al., 1995). The nuclear matrix and intermediate filaments, referred to as the nuclear matrix-intermediate filament complex (NMIF), are integrated into a single structure encompassing the whole cell and have the appearance and structure of an intact cell after extraction of soluble proteins and DNA (Nickerson et al., 1995).

The nucleus is organized into multiple subnuclear compartments including the nucleolus, transcription domains, replication domains, splicing factor domains, PML domains and coiled bodies (Xing et al., 1993; Carter et al., 1993; Nickerson et al., 1995; Lamond and Earnshaw, 1998). The nuclear matrix appears to be the structural support that organizes these functional nuclear compartments for nuclear metabolism (Nickerson et al., 1995). Sites of transcription, replication and RNA processing associate with the nuclear matrix in a punctate subnuclear distribution (Nickerson et al., 1995; Stein et al., 2000b; Lamond and Earnshaw, 1998; Cook, 1999; Berezney et al., 2000; Misteli and Spector, 1998). The nuclear matrix may act as an organizing scaffold for the dynamic assembly and disassembly of transcription factor complexes.
and may contribute to the regulation of gene expression by concentrating and localizing transcription factors in a specific place in the nucleus (i.e., subnuclear foci) (Stein et al., 2000b; Zeng et al., 1997).

RUNX2 was originally defined as an osteoblast specific nuclear matrix protein, NMP-2 (Merriman et al., 1995). Studies by the Stein/Lian laboratory have shown that RUNX1 localizes to the nucleus and associates with the nuclear matrix in a punctate subnuclear distribution in fixed cells using immunofluorescence microscopy (Zeng et al., 1997). Two trafficking signals are necessary for the subnuclear targeting of RUNX proteins, one is a nuclear localization signal (NLS), which is located C-terminal to the RHD and is required for nuclear import, and the second is a 31 or 38 amino acid nuclear matrix targeting signal (NMTS) (Zeng et al., 1997; Zaidi et al., 2001; Stein et al., 1998). The NMTS is a conserved sequence in the three RUNX proteins and is located in the C-termini of RUNX factors (amino acids 351-381 of RUNX1/AML-1B and amino acids 397-434 of RUNX2/til-1) (Figure 5A; Zeng et al., 1997; Zaidi et al., 2001). The NMTS has been characterized to be necessary and sufficient for RUNX proteins to associate with the nuclear matrix by immunofluorescence microscopy and biochemical fractionation experiments (Zeng et al., 1997; Zaidi et al., 2001). These results show that RUNX1/AML-1B, but not a C-terminal deleted AML-1B (amino acids 1-290), is associated with the nuclear matrix. Moreover, proteins in which the NMTS sequence is fused to either AML-1B 1-290 (AML-1B 1-290/351-381) or the GAL4 DNA binding domain are associated with the nuclear matrix, suggesting that the NMTS is necessary and sufficient to target RUNX1 to the nuclear matrix (Zeng et al., 1997). These findings using
immunofluorescence microscopy are based on fixed cell preparations and it has been argued that punctate foci observed in nuclear matrix preparations may be an artifact from the fixation or extraction procedures (Pederson, 2000). Therefore, it would be interesting to determine the subnuclear distribution of RUNX factors in living cells.

A subset of RUNX1 foci are associated with sites of active transcription on the nuclear matrix (Zeng et al., 1998). The crystal structure of the RUNX1 NMTS reveals a two-finger structure model, which is composed of two loops connected by a flexible U-shaped peptide (Figure 5B; Tang et al., 1999). It was thought that the NMTS might interact with the nuclear matrix at the tips of the loops (Tang et al., 1999). Deletion of the C-terminus of RUNX1 or RUNX2, which removes the NMTS and several co-regulatory interaction domains, results in a loss of nuclear matrix association and a reduction in transactivation of the TCRβ enhancer and osteocalcin (OC) promoter, respectively (Zeng et al., 1997; Zaidi et al., 2001; Tang et al., 1999). Furthermore, deletion of the C-terminus of RUNX2 in gene-replacement mice, which introduced a stop codon at amino acid 376 before the NMTS and several co-regulatory protein interaction domains, resulted in a phenotype similar to RUNX2 null mice, lack of bone formation and maturation of osteoblasts, indicating that the C-terminus of RUNX2 is required for osteogenesis (Choi et al., 2001). Therefore, these findings suggest a functional role for nuclear matrix association in the regulation of tissue-specific gene expression.
Figure 5. Sequence and Crystal Structure of the Nuclear Matrix Targeting Signal (NMTS). (A) The sequence of the NMTS is shown for RUNX1 (AML-1B), RUNX2 (AML3) and RUNX3 (AML2). The NMTS is highly conserved between RUNX proteins in several different species. Conserved amino acids are highlighted. (Adapted from Zeng et al. (1997). *PNAS* 94, 6746-51.) (B) The crystal structure of the RUNX1 NMTS is illustrated. The NMTS structure shows two loop domains, Loop I and Loop II, separated by a glycine-rich α-helical turn. (Adapted from Tang et al. (1999). *J. Biol. Chem.* 274, 33580-6.)
A

RUNX1
AML1 human 233 RNYPGAG---S-L-HGSS---AT-KR-PPPPYTG 274
mouse
rat

RUNX2
AML3 human 238 RNYPAT---S-L-HGSS---AT-KR-PPPPYTG
mouse

RUNX3
chicken

Nuclear Matrix Targeting Signal

B

Loop I
238
250

Loop II
260
268

Strand II

Turn
255
Functional Relationship between Gene Regulation and Nuclear Architecture:
Spatial and Temporal Targeting of RUNX Proteins

The interrelationship between gene regulation and nuclear matrix association is an evolving concept. The association of genes and transcription factors with the nuclear matrix at subnuclear domains may facilitate transcriptional control (Stein et al., 1998). The ability to regulate transcription and associate with the nuclear matrix is different for the RUNX deletion mutants. RUNX2ΔC, which lacks the C-terminus of RUNX2, is not associated with the nuclear matrix in calvarial cells extracted from homozygous RUNX2ΔC mutant knock-in mice, whereas in calvarial cells from wild-type mice RUNX2 is associated with the nuclear matrix (Choi et al., 2001). A decrease in the transactivation of the OC promoter is observed in HeLa cells after removal of the RUNX2 C-terminus (Choi et al., 2001). Furthermore, the full-length RUNX1 isoform, AML-1B, contains a promoter-context dependent transactivation domain and is associated with the nuclear matrix (Zeng et al., 1997; Lutterbach and Hiebert, 2000). Conversely, a C-terminal truncated RUNX1 protein (AML-1B 1-290) and the AML-1 isoform are transcriptionally inactive and not associated with the nuclear matrix (Zeng et al., 1997; Speck and Stacy, 1995). Taken together, these results suggest that there is functional relationship between gene regulation and nuclear matrix association.

Directing nuclear proteins to the correct place in the nucleus is important for proper control of transcription (Stein et al., 1998). The t(8;21) chromosomal translocation results in the fusion of the RUNX1/AML-1 gene after the RHD with the ETO (eight-twenty-one) or MTG8 gene creating the protein product AML-1/ETO
(see Figure 4; Erickson et al., 1992; Miyoshi et al., 1993), which lacks the RUNX1 subnuclear targeting signal (the NMTS) and co-regulatory protein interaction sites. The Stein/Lian laboratory has shown that AML-1/ETO is not targeted to RUNX1/AML-1B subnuclear sites (McNeil et al., 1999). Instead, AML-1/ETO is redirected to ETO nuclear matrix associated foci. The 8;21 translocation of the RUNX1/AML-1 gene in acute myelogenous leukemia modifies the intranuclear trafficking of RUNX1/AML-1B proteins by the misrouting of RUNX1/AML-1B to alternate subnuclear sites (McNeil et al., 1999). This result suggests that localization to the right place in the nucleus is very important for proper cellular function.

**Green Fluorescent Protein**

Bioluminescence is common in many marine invertebrates, including several cnidarians and ctenophores. Light emitted from cnidarians is primarily green, whereas that from ctenophores is blue. Green fluorescent protein (GFP) was originally discovered as a glowing green fluorescence in the jellyfish *Aequorea victoria*, which emits light as a defense mechanism (Prasher et al., 1992). In vivo, GFP is excited when calcium ions bind to the photoprotein aequorin resulting in the emission of blue light (Prasher et al., 1992; Chalfie et al., 1994). The energy emitted from the aequorin proteins is then transferred to the GFP molecule, which is then excited and emits green light. Wild-type GFP has a major excitation peak of 395 nm and a minor peak at 475 nm and gives an emission peak at 508 nm (Tsien, 1998).

After many years of collecting jellyfish from the ocean and painstakingly extracting GFP protein to be used in experimental studies, this task was facilitated
when GFP from the *Aequorea victoria* jellyfish was cloned and sequenced (Prasher et al., 1992) and when GFP was demonstrated to express in eukaryotic and prokaryotic cells (Chalfie et al., 1994). GFP was determined to be a 238-amino acid protein with a molecular mass of 27 kDa (Prasher et al., 1992). Expression vectors for GFP became readily available after GFP was cloned. GFP could be expressed in living cells and excited by fluorescence microscopy with blue light resulting in the emission of green light from the GFP molecule.

GFP must be properly folded in order to emit the green light (Tsien, 1998). The region of GFP that is responsible for the emission of green light is the chromophore, which consists of the hexapeptide from amino acids 64-69 (Figure 6A; Prasher et al., 1992). Cyclization of the trimer Ser-dehydroTyr-Gly (amino acids 64-67) in the chromophore is required for light emission (Cody et al., 1993). For the cyclization mechanism, GFP folds into a nearly native conformation and the imidazolinone is formed by nucleophilic attack of the amide group of Gly67 on the carbonyl group of Ser65 followed by dehydration and oxidation of Tyr66 (Figure 6B; Tsien, 1998). The crystal structure of GFP shows a barrel structure of eleven β-sheets surrounding the helical chromophore, which has been referred to as 'paint in a can' (Figure 6C; Ormo et al., 1996; Yang et al., 1996a). Two GFP β-barrel monomers can homodimerize (Ormo et al., 1996; Yang et al., 1996a).

Multiple variations of GFP have been generated to change its color, to increase its intensity and to increase the expression of properly folded fluorescent protein at 37°C (Yang et al., 1998). Several different colored fluorescent proteins have been created by generating point mutations in GFP yielding proteins with shifted
Figure 6. Structure of Green Fluorescent Protein and its Chromophore. (A) The chromophore of Green Fluorescent Protein (GFP) is the region of the protein responsible for emitting green light and is a hexapeptide from amino acids 64-69. Cyclization of the trimer Ser-Tyr-Gly (amino acids 65-67) in the chromophore is necessary for the fluorescence of GFP. The cyclic Ser-dehydroTyr-Gly structure is shown. (B) The mechanism to create the cyclic Ser-dehydroTyr-Gly structure is to first fold GFP into a nearly native conformation, then cyclization occurs by the formation of an imidazolinone group by nucleophilic attack of Gly67 on Ser65 followed by dehydration and oxidation of the cyclic group of Tyr66. (C) The crystal structure of GFP reveals a β-barrel structure, which is very stable. The β-barrel contains 11-β-sheets surrounding an α-helical chromophore in the center.
A  Cyclic GFP Chromophore


B  The Cyclization Mechanism of the GFP Chromophore

C

Crystal Structure of GFP

emission spectra, including cyan fluorescent protein (CFP), which has major and minor excitation and emission peaks at 433 (453) nm and 475 (501) nm, yellow fluorescent protein (YFP), which has excitation and emission maxima at 513 nm and 527 nm, and blue fluorescent protein (BFP), which has excitation and emission peaks at 380 nm and 440 nm. The long desired red fluorescent protein (drFP583) was cloned from the Anthozoa species of coral and has an absorption peak at 558 nm and an emission peak at 583 nm (Matz et al., 1999). However, red fluorescent protein has the disadvantage that it can form protein aggregates when expressed in cells as a fusion protein. One variant of GFP is enhanced green fluorescent protein (EGFP; Clontech, Palo Alto, CA), which is mammalian codon optimized for increased translational efficiency in mammalian cells and has Ser65Thr and Phe64Leu mutations that increase its brightness 35-fold. EGFP has an excitation peak at 488 nm and an emission peak similar to wild-type GFP at 509 nm (Yang et al., 1996b; Cormack et al., 1996). In this study, we have used EGFP fused to RUNX factors to assess the intranuclear trafficking of RUNX proteins in living cells.

Several advantages of using GFP are that it has an intrinsic fluorescence in which no antibodies, substrates or cofactors are required, it is very stable, does not photobleach easily, it is species independent, and can be monitored in living cells and whole animals (Kain et al., 1995). GFP has several applications including as a protein tag, monitoring gene expression in living cells and tissues, drug and genetic screens, and studying protein dynamics in subcellular compartments (Misteli and Spector, 1997; Gerdes and Kaether, 1996). Tagging nuclear proteins with GFP to determine their mobility has recently advanced the way we think of the nucleus.
Protein Dynamics

Determining the mobility of proteins and lipids in membranes, such as the plasma membrane, the mitochondrial inner membrane and liposomes, began 25 years ago using Fluorescence Recovery After Photobleaching (FRAP) analysis by microinjection of proteins and lipids labeled with fluorophores and fluorescent antibodies (Axelrod et al., 1976). FRAP has several other names such as fluorescence photobleaching recovery, fluorescence photobleaching redistribution (FPR) and fluorescence microphotolysis. Most recently, GFP has been fused to proteins to determine their mobility using FRAP since GFP exhibits much less photodamage to cells than other fluorophores (Spector et al., 1998).

In a typical FRAP assay, an image is captured using confocal microscopy before photobleaching and then the fluorescence in a small area of the cell is photobleached using a short pulse of a high-powered laser. The recovery of the fluorescence resulting from movement of the unbleached molecules into the photobleached area is determined by capturing images at certain time intervals after photobleaching (Spector et al., 1998). Two parameters can be calculated from FRAP analysis: 1) the mobile fraction of the fluorescent molecules; and 2) the rate of the mobility (Reits and Neefjes, 2001). The mobile fraction and the rate of mobility are measurements of the exchange of the unbleached molecules outside the photobleached area for the bleached molecules inside the photobleached area. The calculations of the mobile fraction and rate of the mobility are assessed from the graph of the recovery curves (fluorescence intensity vs. time) (Spector et al., 1998).
FRAP analysis can determine the mobility of cytoplasmic and nuclear proteins in addition to proteins that are membrane bound. The mobility of proteins in the nucleus may reflect a diffusional mechanism, which does not require energy or a directional mechanism, which may be targeted by specific signals (Misteli, 2001). Nuclear proteins that collide with other proteins or with the nuclear architecture may move more slowly than freely diffusing proteins (Misteli, 2001). Therefore, FRAP analysis provides a very useful tool to determine the dynamics of nuclear proteins.

The Present Study

Previous results have shown that RUNX factors have a punctate subnuclear distribution and that the nuclear matrix targeting signal (NMTS) of RUNX proteins is necessary to associate with the nuclear matrix in fixed cells (Zeng et al., 1997; Zaidi et al., 2001; Tang et al., 1999). However, the localization of RUNX proteins in living cells is unknown. Therefore, in this study we determined the subnuclear distribution of RUNX proteins in living cells and whether the subnuclear distribution is dependent on the C-terminus, which contains the NMTS. To assess whether RUNX nuclear proteins move through the nucleus quickly or slowly to the subnuclear domains and whether the mobility of RUNX proteins is constrained by the nuclear matrix, we examined the dynamics of RUNX proteins in living cells using fluorescence recovery after photobleaching (FRAP) analysis and determined the effect of deleting the C-terminus and mutating the nuclear matrix targeting signal (NMTS) on the mobility of RUNX2. To understand the subnuclear organization of RUNX proteins in live cells, we assessed whether there are a fixed number of sites in the nucleus or whether the
number of sites is variable and whether RUNX sites are saturable after forced
description of RUNX proteins. Furthermore, since the subnuclear targeting signals in
the three RUNX proteins are conserved (Zeng et al., 1997), we assessed whether
RUNX1 and RUNX2 are directed to the same subnuclear sites in the nuclear matrix.
These studies will provide insight into the formation and maintenance of RUNX
subnuclear sites and the intranuclear trafficking of RUNX transcription factors into
and out of subnuclear foci.

Overall Goal

The overall goal of this study is to assess the intranuclear trafficking of and
the possible mechanism by which RUNX transcription factors move to subnuclear
domains in living cells to understand the spatial-temporal localization of RUNX
proteins.

Hypothesis

We hypothesize that RUNX transcription factors dynamically move through
the nucleus and associate with subnuclear domains in a C-terminal dependent
mechanism to regulate transcription. The biological questions we address are: 1)
What are the subnuclear distribution and dynamics of RUNX proteins in living cells
and the effect of the C-terminus of RUNX on the mobility; 2) What effect does
mutation of the nuclear matrix targeting signal have on the subnuclear distribution
and mobility of RUNX transcription factors; and 3) Are RUNX subnuclear foci
saturable and are RUNX1 and RUNX2 targeted to common subnuclear domains?
CHAPTER 1

Transcription Factors RUNX1/AML1 and RUNX2/Cbfa1 Dynamically Associate with Stationary Subnuclear Domains in a C-terminal Dependent Mechanism
INTRODUCTION

Regulatory factors involved in cellular processes that include gene transcription, DNA replication and RNA processing, are localized to distinct domains in the nucleus (Nickerson et al., 1995; Stein et al., 2000b; Lamond and Earnshaw, 1998; Cook, 1999; Berezney et al., 2000; Misteli and Spector, 1998). One example is the family of runt-related transcription factors (RUNX), which localize within the nucleus to punctate foci and associate with the subnuclear scaffold designated as the nuclear matrix (Zeng et al., 1997; Zeng et al., 1998; McNeil et al., 1999; Tang et al., 1999; Stein et al., 2000b). A subset of RUNX1 subnuclear foci are involved in transcriptional control as shown by colocalization of RUNX1 foci with sites of active transcription (Zeng et al., 1998). Endogenous and overexpressed RUNX proteins both have a punctate subnuclear distribution and are nuclear matrix associated (Zeng et al., 1997; Tang et al., 1999; Prince et al., 2001).

RUNX1 and RUNX2 each contain a conserved C-terminal nuclear matrix targeting signal (NMTS) that is necessary for directing these proteins to intranuclear foci (Stein et al., 2000b; Zeng et al., 1997; Zaidi et al., 2001). The C-terminus of RUNX factors is also a functional domain for transcriptional modulation by several co-regulatory proteins (Lutterbach and Hiebert, 2000). RUNX proteins are required for tissue-specific gene expression in hematopoiesis (RUNX1) and skeletogenesis (RUNX2) (Okuda et al., 1996; Wang et al., 1996a; Stewart et al., 1997; Komori et al., 1997; Otto et al., 1997; Choi et al., 2001). Recently, knock-in mice which deleted the C-termini of RUNX1 and RUNX2 were used to demonstrate that the C-terminal
domain of either RUNX1 or RUNX2, which contains the NMTS, is essential for hematopoiesis and bone formation, respectively (Choi et al., 2001; North et al., 1999). Thus, the C-terminus of RUNX factors appears to be involved in both intranuclear trafficking and transcriptional regulation.

The dynamics by which proteins traverse and localize within the nucleus may be critical for their biological activity. One important question relates to the relative mobility and compartmentalization of RUNX transcription factors in subnuclear foci in living cells. Mobility studies that began 27 years ago used Fluorescence Recovery After Photobleaching (FRAP) assays to determine the dynamics of proteins and lipids in cellular membranes (Axelrod et al., 1976; Spector et al., 1998). Fluorophores and fluorescent antibodies were conjugated to proteins and lipids and microinjected into cells to determine their mobility. The discovery of green fluorescent protein (GFP), which contained intrinsic green fluorescence, facilitated the use of FRAP analysis by expressing nuclear and cytoplasmic proteins fused to GFP. An emerging concept is that the mobility of proteins is directly coupled to their function and whether they are architecturally linked to specific subnuclear compartments. Association with the nuclear architecture may slow the mobility of nuclear proteins down. The dynamics of RUNX transcription factors may reflect the movement to and from subnuclear foci that are involved with transcription. RUNX may assemble into functional transcription factor complexes at these subnuclear foci to regulate gene expression.

To understand the relative mobility of RUNX transcription factors in the nucleus and the dynamics of their association with subnuclear sites, we used time-lapse microscopy and FRAP analysis. Our key result is that RUNX1 and RUNX2
transcription factors are targeted to and dynamically associate with subnuclear foci that remain stationary within the nuclear space. Furthermore, we show that a C-terminal truncation of RUNX2 that removes the subnuclear targeting signal increases the mobility of the protein to that of EGFP alone and results in the loss of nuclear matrix association and interaction with subnuclear foci. Moreover, we show that RUNX1 and RUNX2 proteins are targeted to common subnuclear domains. RUNX subnuclear foci may represent sites of transcription, which contain co-regulatory factors and nascent transcripts. These findings together with others suggest that the dynamic association of RUNX proteins in stationary foci provides a mechanism for formation of regulatory complexes that are essential for RUNX dependent cell differentiation and embryonic development.
MATERIALS AND METHODS

Cell Culture

Human osteosarcoma SaOS-2 cells (American Type Culture Collection (ATCC), Manassas, VA) were maintained in McCoy’s 5A medium supplemented with 15% (v/v) fetal bovine serum (FBS), human cervical carcinoma HeLa cells (ATCC) were maintained in Dulbecco’s modified eagle medium (DMEM) with 10% FBS and rat osteosarcoma ROS 17/2.8 cells (Majeska et al., 1980) were maintained in F12 media with 5% FBS at 37°C in a humidified 5% CO2 incubator. All media was supplemented with 100 Units/ml Penicillin, 100 µg/ml Streptomycin and 2 mM L-glutamine and changed every other day.

Plasmids

Generally, the Enhanced Green Fluorescent Protein (EGFP; Clontech, Palo Alto, CA) gene was cloned into pcDNA3 (Invitrogen, Carlsbad, CA) and fused to the genes for the full-length mouse PEBP2αA isoform of RUNX2 (amino acids 1-513), the C-terminal deleted PEBP2αA isoform RUNX2Δ361 (amino acids 1-361) and the human AML1B isoform of RUNX1 (amino acids 27-480). EGFP-RUNX2 and EGFP-RUNX2Δ361 were generated by inserting a PCR amplified EGFP cDNA into pcDNA3 and then cloning either RUNX2 or RUNX2Δ361 genes. Specifically, the NheI/EcoRI fragment of the pEGFP-C3 vector (Clontech) was used as a template, and PCR was performed as follows: 5 min at 94°C; 30 cycles of 1 min at 94°C, 3 min at 42°C and 3 min at 72°C; and then extended for 10 min 72°C using the forward
primer 5'-TCTAGAGGTACCAGTGAGCAAGGGC-3' which contains a KpnI/Acc65I restriction site and the reverse primer 5'-ATAGAATTCGGATCCCTTGTACAGCTCGTC-3' with engineered BamHI and EcoRI sites. In a final volume of 50 µl, the PCR reaction contained a final concentration of ~1 ng template DNA, 1X Thermopol Buffer (New England Biolabs, Beverly, MA), 4.5 mM MgSO₄ (New England Biolabs), 0.5 µM of each primer, 0.2 mM dATP, dCTP, dGTP and dTTP mixture (Amhersham Pharmacia, Piscataway, NJ), 10% (v/v) glycerol and 2.5 units of Vent polymerase (New England Biolabs).

The KpnI/Acc65I and EcoRI sites were used to insert the amplified EGFP cDNA fragment into the dephosphorylated pcDNA3 vector. RUNX2 and RUNX2Δ361 genes (Javed et al., 2001) were subsequently subcloned into this plasmid at the 3' end of EGFP using the BamHI site and either XbaI or XhoI sites, respectively.

Similarly, the EGFP-RUNX1 plasmid was generated by inserting a PCR amplified EGFP DNA product into pcDNA3 and then adding the RUNX1 DNA. EGFP was amplified by PCR using the oligonucleotides 5'-GGATCCGGTACCAGTGAGCAAGGGCGAGGAG-3' as the forward primer which contained the generated KpnI/Acc65I site and 5'-GAATTCTCTAGACTTGTACAGCTCGCCATGCC-3' as the reverse primer, in which an XbaI site was created. In a final volume of 50 µl, the PCR reaction contained a final concentration of ~1 ng template DNA, 1X cloned Pfu reaction buffer (Stratagene, La Jolla, CA), 0.5 µM of each primer, 0.2 mM dATP, dCTP, dGTP and dTTP mixture (Amhersham Pharmacia, Piscataway, NJ), 10% (v/v) glycerol and 3.75 Units of Pfu polymerase (Stratagene, La Jolla, CA). The PCR
product was digested with Acc65I and XbaI and then ligated to a similarly digested pcDNA3 vector to generate pcDNA3-EGFP. The XbaI/XbaI fragment of RUNX1 (amino acids 27-480; Zeng et al., 1997) was then inserted into this plasmid. Orientation of the RUNX1 cDNA was determined by digesting with HindIII. All clones were manually sequenced using Sequenase version 2.0 kit (Amersham Pharmacia, Piscataway, NJ).

Restriction Digestion of DNA

Restriction digestions for cloning EGFP-RUNX fusion constructs were slightly different for each construct produced. Briefly, for creating pcDNA3-EGFP, pcDNA3 and the EGFP PCR product were digested in a total volume of 70 µl containing 10 µg DNA or half of (30 µl) the PCR product, 1x Buffer #3 (NEB), 100 µg/ml bovine serum albumin (BSA), and 3 µl Acc65I enzyme (NEB) for 3 hours at 37 °C. The enzyme was heat inactivated at 65 °C for 20 minutes. One microliter of digested DNA was loaded on a 0.7% agarose gel to check for complete digestion. The completely linearized DNA was ethanol precipitated with 5 volumes of 100% ethanol (350 µl) and 1/10 volume of 3 M sodium acetate pH 5.2 (7 µl) on dry ice for 20 minutes, centrifuged at 14,000 rpm for 30 minutes at 4 °C, washed with 70% ethanol, centrifuged at 14,000 rpm for 10 minutes, air dried for 5-10 minutes and resuspended in 20 µl of ddH2O or TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA). The Acc65I digested DNA was then digested with EcoRI in a volume of 30 µl using 1x EcoRI buffer (NEB), and 2 µl of EcoRI enzyme (NEB) for 3 hours at 37 °C. The enzyme was heat inactivated at 65 °C for 20 minutes.
Isolation of Digested DNA Fragments

Digested PCR products and plasmid DNA were loaded on a 0.7% agarose gel. The appropriate size bands were excised with a clean scalpel and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) according to the manufacturer’s suggested protocol. The weight of the gel slice was determined. Three volumes of Buffer QG were added to 1 volume of the gel slice in a 1.5-ml microcentrifuge tube (100 mg~100μl). The gel slice was dissolved at 50 °C for 10 minutes. One volume of isopropanol was added to the sample and mixed. The DNA was bound to the QIAquick gel extraction column by centrifugation of 750 μl of the sample at a time for 1 minute at 14,000 rpm until the entire sample was centrifuged into the column. The flow-through was discarded; 0.5 ml of Buffer QG was added to the column and centrifuged for 1 minute. The column was washed with 0.75 ml of Buffer PE and centrifuged for 1 minute. The flow-through was discarded and the column was spun for an additional 1 minute to remove the residual ethanol from Buffer PE. The column was then placed in a clean microcentrifuge tube and the DNA was eluted with 30-50 μl of Buffer EB (10 mM Tris-HCl, pH 8.5) or ddH2O and centrifuged for 1 minute.

Dephosphorylation of DNA

Before ligation with the insert, the digested pcDNA3 vector was treated with calf intestine alkaline phosphatase (CIP) to prevent the vector from self-ligating and to reduce the background. Dephosphorylation of the 5’ phosphate group on the DNA was performed in the NEB buffer used for digestion. 1.5 μl of CIP enzyme (NEB; 10
Units/µl) was added to the reaction and incubated at 37 °C for 2 hours. To inactivate the reaction, 3-5 mM EDTA was added and incubated at 75 °C for 20 minutes.

**DNA Ligation Reactions**

Ligation of vector and insert was performed at 1:3 and 1:1 weight ratios (vector: insert). Gel-extracted digested vector and insert DNA (1 µl each) were loaded on a 0.7% agarose gel and the amount of each DNA in 1 µl was estimated by eye. The ligation was performed using ~50 ng of vector, ~50 or 150 ng of insert, 2 µl T4 DNA ligase buffer {50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 mg/ml BSA (NEB)} and 400 Units T4 DNA ligase (NEB) at 22 °C for 3 hours or at 16 °C overnight (~16 hours).

**Transformations**

Competent DH5α, HB101 or XL-10 Gold (Stratagene) bacterial cells were thawed on ice for 10 minutes. Ligated DNA (2 µl; 0.1 volume) was mixed with 50 µl of cells and incubated on ice for 15-30 minutes. Cells were heat shocked at 42 °C for 0.5-2 minutes and placed on ice for 5 minutes. 500 µl of LB without antibiotics was added to the cells and incubated at 37 °C for 1 hour. The cells were centrifuged quickly, resuspended in 50 µl of LB, spread on LB Ampicillin (50-100 µg/ml) plates and incubated at 37 °C overnight for 16 hours.
Small Scale DNA Preparations

An overnight culture (5ml) of Luria-Bertani (LB) medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl) with 50-100 μg/ml of Ampicillin was initiated from 1 colony and grown at 37 °C. Plasmid DNA was isolated using QIAprep spin miniprep kit (Qiagen). Cell pellets (from 3ml of the culture) were resuspended in 250 μl of Buffer P1 (50 mM Tris-Cl, pH 8.0; 10 mM EDTA; 10 μg/ml RNase A). Buffer P2 (250 μl; 200 mM NaOH; 1% SDS) was added to each tube, which was inverted 4-6 times and cells were lysed for 5 minutes at room temperature. Buffer N3 (350 μl; contains guanidine hydrochloride to bind the DNA to the silica in column; Qiagen) was added to the lysed cells and the tube was inverted 4-6 times to mix. The sample was then was centrifuged for 10 minutes to remove the white precipitate formed. The supernatant was loaded on a QIAquick spin column attached to a vacuum manifold and the vacuum was applied to bind the DNA to the column. The column was then washed with 0.5 ml of buffer PB (a wash buffer that contains guanidine hydrochloride and isopropanol; Qiagen) and the vacuum was applied. 0.75 ml of Buffer PE (a low salt buffer containing 80% ethanol; Qiagen) was added to the column and the vacuum was applied. To remove the residual ethanol from Buffer PE, the column was centrifuged for 1 minute at 14,000 rpm. The DNA was then eluted in a new tube with 50 μl of Buffer EB (10 mM Tris-HCl, pH 8.5) or ddH₂O by centrifugation for 1 minute at 14,000 rpm.
Large Scale DNA Preparations

A small culture (5 ml) was first generated from 1 colony and grown for 5-8 hours at 37 °C in a shaking incubator. A large culture (200 ml of LB with 50-100 μg/ml Amp) was then inoculated from the small culture and grown 16 hours at 37 °C in a shaking incubator. The bacterial cells were centrifuged in a Beckman JA-10 rotor at 6,000 rpm for 15 minutes. Plasmid DNA was extracted using the Qiagen Maxiprep kit (Qiagen). Each pellet was resuspended in 10 ml of Buffer P1 containing RNase A and transferred to a 50 ml plastic tube. The cells were lysed with 10 ml of Buffer P2, inverted 4-6 times and incubated for 5 minutes at room temperature. The cell debris was then precipitated with 10 ml of Buffer P3 (3.0 M potassium acetate, pH 5.5), incubated on ice for 20 minutes and centrifuged for 30 minutes at 15,000 rpm in a Beckman JA-20 rotor at 4 °C. The supernatant was transferred to a new 50 ml plastic tube and centrifuged for another 15 minutes at 15,000 rpm. The tip-500 column was equilibrated with 10 ml of Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% Triton X-100). The supernatant was added to the column to bind the DNA. The column was washed 3 times with Buffer QC (1.0 NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol) and the DNA was eluted with 15 ml of Buffer QF (1.25 M NaCl; 50 mM Tris-Cl pH 8.5; 15% isopropanol) in a 30 ml corex tube. 10.5 ml of isopropanol was added to the eluted DNA and the DNA was centrifuged at 10,000 rpm for 45 minutes at 4 °C. The pellet was washed with 5 ml of 70% ethanol and centrifuged at 10,000 rpm for 10 minutes. The DNA was air dried for 5-10 minutes and resuspended in 500 μl of TE, pH 8.0.
Sequencing

Cloned plasmid DNA was sequenced using T7 Sequenase version 2.0 DNA sequencing kit (Amersham Pharmacia, Piscataway, NJ). To denature the DNA, 5 μg of maxiprep DNA was incubated in 12 μl total volume with 333 mM NaOH and 0.333 mM EDTA for 5 minutes at room temperature. 2 M ammonium acetate (2 μl) was added and placed on ice. The DNA was ethanol precipitated with 5 volumes of 100% ethanol, incubated on dry ice for 15 minutes, centrifuged at 14,000 rpm for 15 minutes, washed with 70% ethanol, centrifuged for 10 minutes and air dried on ice for 5-10 minutes. The DNA was resuspended in a 10 μl volume using 20 ng of primer {T7 primer (5'-TAATACGACTCACTATAGGG-3'), Sp6 primer (5'-TATTTAGGTGACACTATAG-3'), SITE C top strand primer (5'-GTCACCAACCACAGCATCCTTTG-3'), 3'EGFP primer (5'-GCAAAGACCCCCAAGC-3') or 5'pcDNA3 primer (5'-GCTAACTAGAGAACC-3') and 2 μl 5X T7 sequencing reaction buffer. To anneal the primers to the DNA, DNA-primer reaction mix was heated at 65°C for 2 minutes and slow cooled on the bench to ~35-25°C. Individual tubes were filled with 2.5 μl of each termination mix (ddG, ddA, ddT, ddC), capped and heated to 37°C. For the each labeling reaction, a mixture containing 1 μl 0.1 M DTT, 2 μl 1X labeling mix, 1 μCi $^{35}$S-αdATP (NEN), 1 Unit inorganic pyrophosphate, and 2 μl of 6X diluted T7 Sequenase version 2.0 polymerase (13 Units/μl undiluted) in enzyme dilution buffer was added to the annealed DNA mixture (3.5 μl) and incubated for 4-10 minutes at 37°C. Stop solution (4 μl) was added to each tube to stop the labeling reaction. Samples were heated to 95°C and loaded (5 μl) on a 6% polyacrylamide Sequagel (National
Diagnostics, Atlanta, Georgia), which was pre-run at 50W for 30 minutes. The gel was dried and exposed to autoradiograph XAR-5 film (Kodak, Rochester, NY) overnight at −70 °C.

**Transient Transfections**

*Lipojectamine Plus:* At 60-90% confluence, SaOS-2 cells were transfected using LipojectAMINE Plus reagents (Invitrogen, Carlsbad, CA). DNA and Plus reagent were pre-complexed in serum-free DMEM for 15 minutes at room temperature. LipojectAMINE reagent was diluted in serum-free DMEM. The pre-complexed DNA-Plus mix was then combined with the LipojectAMINE mix and incubated at room temperature for 15 minutes. Cell cultures were washed with serum-free DMEM and replaced with serum-free DMEM. The DNA-Plus-LipojectAMINE complexes were added to the cells and incubated at 37 °C for 2 hours. Cells were washed twice with serum-free McCoy’s 5A medium and fed with completed McCoy’s 5A medium. The amounts of each reagent, DNA and medium are shown in the table below for each well in 6-well plates, 100 mm plates and T25 flasks.

<table>
<thead>
<tr>
<th>Culture dish</th>
<th>DNA (µg)</th>
<th>PLUS Reagent (µl)</th>
<th>Serum-free Dilution media (µl)</th>
<th>LipofectAMINE Reagent (µl)</th>
<th>Transfection medium (ml)</th>
<th>Transfection volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-well</td>
<td>0.25-1.0</td>
<td>3</td>
<td>100</td>
<td>2.5</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>100 mm</td>
<td>1-4</td>
<td>10</td>
<td>750</td>
<td>15</td>
<td>5</td>
<td>6.5</td>
</tr>
<tr>
<td>T-25 flask</td>
<td>2</td>
<td>4</td>
<td>250</td>
<td>5</td>
<td>2</td>
<td>2.5</td>
</tr>
</tbody>
</table>
**Superfect**: ROS 17/2.8 or HeLa cells were grown to 50-80% confluence. DNA was diluted in serum-free DMEM and Superfect (Qiagen) was added and incubated for 10 minutes at room temperature. Cells were washed with serum-free DMEM. Completed DMEM was added to the complexes and the complexes were incubated with the cells for 2 hours at 37 °C. Cells were washed with serum-free DMEM medium for HeLa cells or serum-free F12 medium for ROS 17/2.8 cells, fed with completed media and incubated overnight at 37 °C. The amounts of Superfect reagent, DNA and medium are shown in the table below for each well in 6-well plates and 100-mm plates.

<table>
<thead>
<tr>
<th>Culture dish</th>
<th>DNA (µg)</th>
<th>Superfect Reagent (µl)</th>
<th>Serum-free Dilution media (µl)</th>
<th>Transfection medium (ml)</th>
<th>Transfection volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-well</td>
<td>1-3</td>
<td>5-8</td>
<td>100</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>100 mm</td>
<td>10-20</td>
<td>40</td>
<td>300</td>
<td>2.7</td>
<td>3</td>
</tr>
</tbody>
</table>

**Transcription Assays**

HeLa cells were plated in 6-well plates at a density of 0.6 x 10⁶ cells per plate and transiently transfected at 50-80% confluence using in each well 5 µl Superfect reagent (Qiagen, Valencia, CA), 500 ng of each expression vector (as shown in Chapter 1, Figure 1D), 50 ng of the minimal osteocalcin (OC) promoter -83-OC-Luciferase (Towler et al., 1994), used as a control for transfection efficiency, and 2.5 µg of the rat -1.1 kb OC promoter-CAT reporter gene (Schepmoes et al., 1991). Reporter activities were determined 36-40 hours following transfection. Cells were lysed with 250 µl of 1X Reporter lysis buffer per well (Promega, Madison, WI) for 30
minutes at room temperature. Chloramphenicol acetyltransferase (CAT) activities were determined using 50 μl of cell lysate, 4 mg/ml acetyl coenzyme A (in H2O), 0.25 μCi 14C-Chloramphenicol, and 0.25 M Tris-HCl pH 7.8 per reaction. CAT reactions were incubated at 37 °C for 5 hours. To stop the reactions, 1 ml of Ethyl acetate was added, vortexed and centrifuged for 1-3 minutes. The top organic layer was transferred to a fresh tube and dried in the hood overnight. Twenty μl of ethyl acetate was used to dissolve the residue, which was spotted on a TLC plate (Whatmann, Clifton, NJ). Thin layer chromatography was performed in 285 ml methanol and 15 ml of chloroform (97:3 ratio) for ~1 hour. CAT activities were determined on the Betascope 630 blot analyzer (Betagen, Mountain View, CA) or the Storm 840 Phosphoimager (Molecular Dynamics, Sunnyvale, CA) and normalized to luciferase values. Luciferase reagent (100 μl) from the Luciferase assay system (Promega Corp., Madison, WI) was added to 10-20 μl of cell lysate to determine luciferase values using a 30 second exposure time in the Monolite TM 2010 luminometer (Analytical Laboratory, San Diego, CA). Significance of the results was assessed using the analysis of variance (ANOVA) test and the error bars are shown as the standard error of the mean (SEM.).

**Western Blot Analysis**

HeLa cells were plated at a density of 0.7 x 10^6 in 100 mm plates and transfected with 10 μg of expression plasmid and 40 μl of Superfect reagent (Qiagen). Cell pellets were collected 20 hours after transfection and lysed in 300 μl of lysis buffer containing 8 M urea, 0.1 M NaH2PO4, 0.1 M Tris-HCl, pH 8.0 and a cocktail
of protease inhibitors including, 1.2 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 
μg/ml leupeptin, 0.7 μg/ml pepstatin, 10 μg/ml trypsin inhibitor, 2 μg/ml TPCK, 40 
μg/ml bestatin, 17 μg/ml calpain inhibitor I, and 1 μg/ml E64 (Roche, Indianapolis, 
IN). For each sample 20 μg of total proteins were separated on a 10% SDS-PAGE 
gel. EGFP proteins were detected using a mouse GFP monoclonal antibody 
(Clontech; 1:10,000 dilution). RUNX proteins were detected with rabbit polyclonal 
antibodies to either RUNX2 (1:10,000 dilution) or RUNX1 (1:3,000 dilution) kindly 
provided by Scott Hiebert (Meyers et al., 1996). Appropriate HRP-conjugated 
secondary antibodies (1:10,000 dilution) were detected using the Renaissance 
chemiluminescence kit (NEN, Boston, MA). Cdk2 protein was detected using a rabbit 
polyclonal α-cdk2 antibody (1:5,000 dilution) as a control for protein loading. The 
HRP-conjugated secondary antibodies and the cdk2 antibody were purchased from 
Santa Cruz Biotechnology (Santa Cruz, CA).

In situ Immunofluorescence

SaOS-2 or ROS 17/2.8 cells were grown on 0.5% (w/v) gelatin-coated 
coverslips and cultured to 70% confluency. SaOS-2 cells were transiently transfected 
with 0.25-0.5 μg of expression plasmid, 3 μl Plus reagent and 2.5 μl Lipofectamine 
reagent (Invitrogen, Carlsbad, CA) as above. Cells were harvested 18-20 hours post 
transfection. Whole cell (WC), cytoskeleton (CSK) and nuclear matrix-intermediate 
filament (NMIF) preparations were performed as previously described (Javed et al., 
2000). Briefly, cells were fixed using formaldehyde (3.7%), then permeabilized with
Flowchart A. Schematic of Subcellular Fractionation Methods for In Situ Immunofluorescence Microscopy. Cells were transiently transfected on 0.5% gelatin-coated coverslips in 6-well plates. In each 6-well plate, 2 wells were used for whole cell (WC) preparations, 2 wells for cytoskeleton (CSK) preparations and 2 wells for nuclear matrix-intermediate filament (NMIF) preparations. Briefly, WC cells were fixed with 3.7% formaldehyde and the permabilized with 0.5% Triton X-100. Both CSK and NMIF cells were extracted twice for 15 minutes each with CSK buffer. The CSK prepared cells were then fixed with 3.7% formaldehyde. The DNA in the NMIF prepared cells was digested twice for 30 minutes each with DNase I in digestion buffer. The chromatin was then extracted from NMIF preparations with 250 mM ammonium sulfate. The nuclear matrix-intermediate filament scaffold including insoluble proteins and RNA remains and was fixed with 3.7% formaldehyde. Proteins were then detected with specific antibodies when appropriate.
Subcellular Fractionation Methods for In Situ Immunofluorescence Microscopy

Intact Cell

1. 3.7% formaldehyde then 0.5% Triton

Permabilized Whole Cell Preparation (WC)

1. 3.7% formaldehyde

Cytoskeleton Preparation (CSK)

1. DNase I (2 x 30 min) then
2. 250 mM ammonium sulfate (5 min then 2 min)

Insoluble proteins and RNA (nuclear matrix scaffold)

1. 3.7% formaldehyde

Nuclear matrix-intermediate filament Preparation (NMIF)
0.5% Triton X-100 for whole cell preparations (Flowchart A). CSK and NMIF preparations were extracted twice for 15 minutes each with CSK buffer (100 mM NaCl, 0.3 M sucrose, 10 mM pipes, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, pH 6.8, 2 mM Vanadyl Ribonucleoside Complex (VRC), 0.8 mM 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF)). CSK preparations were fixed in 3.7% formaldehyde in CSK buffer and NMIF preparations were digested twice for 30 minutes each with 400-600 Units/ml of RNase-free DNase I (Roche, Indianapolis, IN) in digestion buffer (CSK buffer with 50 mM NaCl). NMIF prepared cells were extracted with 0.25 M ammonium sulfate in digestion buffer for 10 minutes and fixed with 3.7% formaldehyde in digestion buffer for 10 minutes. Xpress (XPR)-tagged RUNX2 was detected using a primary monoclonal α-mouse Xpress antibody (Invitrogen; 1:800 dilution) and a Texas red-conjugated donkey α-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:200 dilution). Endogenous RUNX2 was detected in SaOS-2 and ROS 17/2.8 cells using a rabbit polyclonal RUNX2 antibody (Oncogene, San Diego, CA; Cat # PC287L; 1:200 dilution, 2.5 ng/ml final concentration) and an Alexa-595 nm goat α-rabbit secondary antibody (Molecular Probes, Eugene, OR; 1:500 dilution). CBFβ was detected using a mouse monoclonal CBFβ antibody (Wang et al., 1996b; 1:20 dilution). Endogenous Sp1 was detected in ROS 17/2.8 cells using the mouse monoclonal Sp1 (IC6) antibody (Santa Cruz Biotechnologies, Santa Cruz, CA; Cat # sc-420; 1:100 dilution). Cells were mounted in Vectashield antifade mounting media (Vector Laboratories, Burlingame, CA). Fluorescence and transmitted light images were captured using a Zeiss Axioplan 2 microscope with a 63X Zeiss Plan-Apochromat objective (1.4
A 100 W Hg lamp and a Hamamatsu digital charged-couple device (CCD) camera interfaced with the MetaMorph Imaging System (Universal Imaging Corp., Downingtown, PA). Cells were counted for extent of colocalization between either RUNX1 and RUNX2 or RUNX2 with two different tags using a dual band pass fluorescence filter (Chroma Technology Corp., Brattleboro, VT, #51006). The software applications used to prepare digital images were Adobe Photoshop and Microsoft PowerPoint.

**BrUTP Labeling**

SAOS-2 cells were transfected with 0.25 μg EGFP-RUNX2 as described above and labeled for BrUTP incorporation 18-20 hours following transfection. Cells were incubated for 3 minutes with glycerol buffer (20 mM Tris-HCl pH 7.4, 5 mM MgCl₂, 0.5 mM EGTA, 25% glycerol) and then for 3 minutes with glycerol buffer supplemented with 0.05% Triton X-100 and 4 mM AEBSF. Nascent transcripts were labeled with BrUTP for 30 minutes at room temperature in transcription buffer (2X Synthesis buffer (100 mM Tris-HCl pH 7.4, 20 mM MgCl₂, 1 mM EGTA, 200 mM KCl, 50% glycerol), 25 μM S-adenosylmethionine (SAM), 500 μM each of ATP, CTP and GTP (Roche, Indianapolis, IN), 750 μM BrUTP (Sigma, St. Louis, MO), 4 mM AEBSF and 40 Units/ml RNase Inhibitor (Roche)). NMIF extractions were performed on cells as described above. A rat monoclonal α-BrdU antibody (Accurate Chemical and Scientific Corp., Westbury, NY; 1:20 dilution) and an Alexa-568 α-rat secondary antibody (Molecular Probes, Eugene, OR; 1:500 dilution) were utilized to detect BrUTP labeling. A Leica SP1 laser scanning confocal microscope interfaced
with Scanware software and a Leica 100X Plan Apo 1.4 N.A. objective were used to capture confocal images. Images were taken using an average of 4-6 sections per cell and 0.365 microns per section. The line scan function in MetaMorph was used to show points of colocalization in a particular area of the nucleus. The extent of colocalization was determined using the colocalization function in MetaMorph (mean ± SEM).

**Time-Lapse Imaging**

SaOS-2 cells were plated at a cell density of $2 \times 10^6$ in 100 mm plates containing gelatin-coated 40 mm coverslips (Bioptechs, Butler, PA). Cells were then transiently transfected using 4 μg of either EGFP, EGFP-RUNX1, EGFP-RUNX2, or EGFP-RUNX2Δ361 expression plasmids, 10 μl Lipofectamine reagent and 20 μl Plus reagent (Invitrogen, Carlsbad, CA). Mitochondria were stained 15-18 hours following transfection with 100 nM of Mitotracker Red CM-H2XRos dye (Molecular Probes, Eugene, OR) in pre-warmed completed McCoy’s 5A media for 30 minutes at 37°C. The Mitotracker Red dye was used as a marker for viability before and after capturing images. Coverslips were then assembled into the FCS-2 closed cell chamber (Bioptechs, Butler, PA) in which a peristaltic pump (Instech Laboratories Inc, Plymouth Meeting, PA) was used to perfuse complete L-15 media without phenol red (Life Technologies) and 10 nM Mitotracker Red dye through the chamber. Cells were maintained at 37°C using the chamber controller and objective heater controller (Bioptechs). Time-lapse images were captured every 10-30 seconds for 20-30 minutes using the Zeiss Axioplan 2 microscope and a 63X Zeiss Plan-Apochromat.
objective with a 1.4 N.A. Exposure times for EGFP fusion proteins were 100-200 milliseconds and for the Mitotracker Red dye were 100-500 milliseconds. Adobe Photoshop, MetaMorph, Microsoft PowerPoint and Adobe Illustrator software were used to prepare the digital images.

**Fluorescence Recovery After Photobleaching (FRAP) Analysis**

SaOS-2 cells were plated in T-25 flasks at a density of $1.2 \times 10^6$ cells/flask and cultured until 70% confluency. Expression plasmids (2 μg) were transiently transfected using 5 μl of Lipofectamine and 4 μl Plus reagents (Invitrogen, Carlsbad, CA). Transfected cells were incubated at 37°C for 6 hours, trypsinized using 1 ml Trypsin-EDTA (Life Technologies) and plated in coverslip live cell chambers. Cells were incubated overnight at 37°C. The Zeiss Axiovert-10 light microscope was used with a Zeiss 100X Plan-Neofluor N.A. 1.30 lens, adapted with a Roper Scientific (Trenton, NJ) cooled CCD camera with a ST-133 controller and an EEV Type 57 back-illuminated frame transfer chip to capture images. Pre-bleached images were captured using a 200 millisecond exposure time. A small area of the nucleus was photobleached using a 476.5 nm Argon ion laser at 100 mW of power for 100 milliseconds. Images of fluorescence recovery were captured every second for 45 seconds using 200 millisecond exposure times.

The half-time of recovery ($t^{1/2}$) was determined by plotting $\ln (i_\infty - i_t)$ vs. time, where $i_\infty$ is the fluorescent intensity at infinity, $i_t$ is the fluorescent intensity in the bleached area at time (t) and then was calculated as $t^{1/2} = \ln 2 \times t_c$, where $t_c = (-1/slope)$
for the punctate distributions. Alternatively for the diffuse distributions, $t_e$ was calculated from the formula:

$$1 - \left(\frac{I_{in}}{I_{out}}\right) = e^{-t/t_e}$$

where, $I_{in}$ is the intensity at time $t$ in the photobleached area, and $I_{out}$ is the intensity at time $t$ outside the photobleached area. The % immobile fraction ($F$) was calculated using the formula:

$$F = \frac{i_{pre} \left( \frac{I_{post}}{I_{pre}} \right)^{1 - I_{in}}}{i_{pre} \left( \frac{I_{post}}{I_{pre}} \right)^{-I_{in}} + e^b}$$

The ratio ($I_{post}/I_{pre}$), where $I_{pre}$ is the pre-bleached intensity over the whole cell and $I_{post}$ is the post-bleached intensity of the whole cell, was used to correct for the extent of photobleaching. $i_{pre}$ is the fluorescent intensity in the pre-bleached area of the nucleus and $b$ is the $y$-intercept of the graph $\ln (I_{in}-I_0)$ vs. $t$. We calculated the recovery rates for both the entire photobleached area and for specific foci in the bleached area. Adobe Photoshop and Microsoft PowerPoint were used to assemble the digital images. Standard errors were determined as the standard error of the mean (SEM).

**Movies on Supplementary CD**

QuickTime Movies 1A-D (available on CD accompanying thesis or at jcs.biologists.org/supplemental) show the time-lapse images corresponding to Figures
7A-D. Time-lapse images were captured for Movie 1A, every 20 seconds for 20 minutes; Movie 1B, every 10 seconds for 30 minutes; Movie 1C, every 20 seconds for 30 minutes; and Movie 1D, every 30 seconds for 30 minutes. QuickTime Movies 2A-D show cells corresponding to those captured in Figure 8A-D for FRAP analysis. Movie 2D (EGFP alone) shows sequential images captured before bleaching and for every second for the first 10 seconds after photobleaching. Movies 2A-C (EGFP-RUNX fusion proteins) show sequential images captured before bleaching and every second for 45 seconds after photobleaching.
RESULTS

Generation of Functional EGFP-RUNX Fusion Proteins

To assess intranuclear dynamics of RUNX proteins in living cells, we prepared a panel of Enhanced Green Fluorescent Protein (EGFP)-RUNX fusion proteins (Figure 1A). We first determined their expression levels and functionality in subnuclear targeting and transcription assays. EGFP was fused to full-length human RUNX1 (amino acids 27-480), mouse RUNX2 (amino acids 1-513) and C-terminal deleted RUNX2Δ361 (amino acids 1-361) (Figure 1A). Western blot analysis with antibodies against EGFP, RUNX1 and RUNX2 using whole cell lysates from transfected HeLa cells demonstrates that EGFP-RUNX fusion proteins are expressed at the expected molecular masses (Figure 1B and C). Cdk2 antibody was utilized as a control for protein loading (Figure 1B and C). Functional activity of the EGFP-RUNX fusion proteins was determined by assessing chloramphenicol acetyl transferase (CAT) reporter gene activity under control of the RUNX-dependent osteocalcin (OC) promoter, which contains three RUNX binding sites (Figure 1D). Transient transfections with RUNX expression vectors were performed with HeLa cells because these cells do not produce endogenous RUNX proteins. EGFP-RUNX1 and EGFP-RUNX2 induce activity of the OC promoter by 12 to 15-fold, whereas non-tagged RUNX1 and RUNX2 produce a 4 to 8-fold activation (Figure 1D). The modest differences in transcriptional induction reflect the higher levels of the EGFP-tagged RUNX proteins relative to the native RUNX proteins (compare Figures 1C and 1D). EGFP alone does not induce OC promoter activity, proving that this protein
Figure 1. Structure and Expression of Functionally Active EGFP-RUNX Fusion Proteins. (A) Schematic of EGFP-RUNX expression constructs. EGFP proteins were fused to the N-termini of RUNX1, RUNX2 and RUNX2Δ361. These constructs were generated using the restriction sites listed above each diagram as described in the Materials and Methods. EGFP-RUNX2Δ361 lacks the C-terminal 152 amino acids of RUNX2, which removes the NMTS, but retains the RHD and NLS. Conserved functional domains of the fusion proteins are labeled as follows: EGFP: Enhanced Green Fluorescent Protein; QA: Glutamine-Alanine amino acid stretch, specific to RUNX2; RHD: Runt Homology Domain; NLS: Nuclear Localization Signal; NMTS: Nuclear Matrix Targeting Signal; VWRPY: conserved interacting sequence for TLE/Groucho, a repression protein.
Figure 1. Structure and Expression of Functionally Active EGFP-RUNX Fusion Proteins. (B and C) Western blot analyses are shown of HeLa cells extracts after transfection with either EGFP-RUNX1, EGFP-RUNX2, EGFP-RUNX2Δ361, or EGFP constructs. pcDNA3 empty vector, CMV-RUNX1 and CMV-RUNX2 expression vectors were used as controls. Proteins were detected using either a monoclonal EGFP antibody (B, top), a polyclonal RUNX1 antibody (C, right) or a polyclonal RUNX2 (C, left) antibody. Cdk-2 antibody was used as a control for equal protein loading (B and C, bottom panels). Positions of molecular weight markers are indicated on the right side of each blot. (D) CAT activity was assessed from HeLa cell extracts co-transfected with each reporter construct (OC promoter-CAT reporter construct and -83-OC-LUC construct) and each expression vector (EGFP, EGFP-RUNX2, EGFP-RUNX2Δ361, RUNX2, EGFP-RUNX1 or RUNX1) as indicated. CAT values were normalized to the luciferase values and fold induction was calculated as CAT activity over the empty vectors. Results are means of 15 to 21 samples ± SEM p<0.0001.
is transcriptionally inert. Deletion of the C-terminus of RUNX2 (EGFP-RUNX2Δ361) reduced transactivation of the OC promoter by 3-fold (Figure 1D). This result is in agreement with previous studies showing that the C-terminus of RUNX2 is required for transcriptional activity (Javed et al., 2000; Zaidi et al., 2001). Taken together, these results show that the EGFP moiety does not interfere with the transcriptional activity of RUNX proteins.

To determine the subnuclear localization and distribution of the EGFP-RUNX fusion proteins, we expressed the fusion proteins in SaOS-2 cells and examined their subnuclear distribution by fluorescence microscopy (Figure 2). Cells were analyzed following either detergent extraction (whole cell, WC) or high salt extraction and nuclease digestion of chromatin (nuclear matrix-intermediate filament preparations, NMIF). The results show that EGFP-RUNX1 and EGFP-RUNX2 proteins produce a punctate nuclear pattern (Figure 2, WC). Both proteins remain in the nucleus following the removal of soluble proteins and chromatin (NMIF). In contrast, the EGFP-RUNX2Δ361 protein, which lacks the subnuclear targeting signal, produces a diffuse fluorescence pattern in both the cytoplasm and the nucleus (WC). Moreover, EGFP-RUNX2Δ361 does not remain in the NMIF fraction, indicating that it is not associated with the nuclear matrix, which is consistent with the observations in Zaidi et al. (2001). Taken together with our previous findings, these results indicate that our panel of EGFP-RUNX fusion proteins is functional, and that the EGFP tag does not interfere with the subnuclear targeting of RUNX proteins.

Since EGFP-RUNX1 and EGFP-RUNX2 were localized into punctate subnuclear foci and were associated with the nuclear matrix, we assessed the
Figure 2. Absence of Subnuclear Organization of a Mutant RUNX Protein in Fixed Cells. SaOS-2 cells were transfected with either the wild-type EGFP-RUNX1, EGFP-RUNX2 or the mutant EGFP-RUNX2Δ361 expression vectors. Both whole cell (WC) and nuclear matrix-intermediate filament (NMIF) preparations were performed as described in the Materials and Methods and show punctate foci for RUNX1 and RUNX2. The green fluorescence of EGFP was captured with a FITC filter (center images). Inserts show DAPI stained nuclei (top left corners) and transmitted light photographs (lower right corners) of each cell. The scale bar equals 10 μm.
localization of endogenous RUNX2 protein by in situ immunofluorescence microscopy. Untransfected SaOS-2 cells were stained with a rabbit polyclonal RUNX2 antibody (Oncogene, San Diego, CA; 1:200 dilution, 2.5 ng/ml final concentration). The results show that endogenous RUNX2 is retained in the nuclear matrix and is expressed in punctate subnuclear foci (Figure 3). We note that it is not possible to assess colocalization of EGFP-RUNX2 and endogenous RUNX2 because the RUNX2 antibodies do not discriminate between exogenous and endogenous RUNX proteins. These results indicate that endogenous RUNX proteins have a similar subnuclear distribution as EGFP-tagged RUNX proteins (compare Figure 3 to Figure 2 in Chapter 1).

RUNX1 and RUNX2 Are Targeted to Common Subnuclear Domains

RUNX1 and RUNX2 have analogous subnuclear targeting signals in their C-termini that are highly conserved (Zeng et al., 1997; Zaidi et al., 2001). Therefore, in situ immunofluorescence microscopy was used to assess whether RUNX1 and RUNX2 are directed to the same subnuclear domains. We also examined as a control whether RUNX2 proteins with different epitope tags are localized in the same subnuclear foci. EGFP- and Xpress (XPR)-tagged RUNX2 proteins (Figure 4A) or EGFP-RUNX1 and XPR-RUNX2 proteins (Figure 4B) were co-expressed in SaOS-2 cells and their subnuclear distribution in whole cell (WC) and nuclear matrix-intermediate filament (NMIF) preparations was monitored. XPR-tagged RUNX proteins were visualized using a Texas red-conjugated secondary antibody and this signal was compared with the intrinsic green fluorescence of EGFP-RUNX proteins.
Figure 3. Endogenous RUNX2 has a Punctate Subnuclear Distribution and Is Associated with the Nuclear Matrix. Whole cell (WC) and nuclear matrix-intermediate filament (NMIF) extractions were performed in untransfected SaOS-2 cells. Endogenous RUNX2 was labeled with a rabbit polyclonal RUNX2 antibody (Oncogene; 2.5 ng/ml final concentration) and an Alexa-595 nm goat α-rabbit secondary antibody (Molecular Probes; 1:500 dilution). DNA was stained with 0.05 μg/ml DAPI.
Figure 4. RUNX1 and RUNX2 Colocalize in Common Subnuclear Domains.

SaOS-2 cells were co-transfected with 0.5 μg each of (A) EGFP-RUNX2 and Xpress (XPR)-RUNX2 (control) and (B) EGFP-RUNX1 and XPR-RUNX2. Whole cell (WC) and nuclear matrix-intermediate filament (NMIF) preparations were performed. The yellow fluorescence in the merged images indicates colocalization between the EGFP- and XPR- tagged RUNX proteins. Cells were stained with 0.05 μg/ml DAPI. Chromatin-extracted NMIF preparations do not present any DAPI staining as expected. Scale bars equal 10 μm. (C) Endogenous RUNX2 (green) and Sp1 (red) transcription factors in WC preparations of ROS 17/2.8 cells were detected using a rabbit polyclonal RUNX2 antibody and a mouse monoclonal Sp1 antibody, respectively. The merged image shows the RUNX2 and Sp1 are not colocalized (lack of yellow fluorescence). Images for RUNX2, Sp1 and Merged were deconvoluted by 2-dimensional nearest neighbor method using MetaMorph imaging software. Localization of RUNX2 and Sp1 in Figure 4C was performed by S. Kaleem Zaidi.
Randomly selected transfected cells (50) were quantitated and evaluated for the extent of signal overlap. The analysis included only those cells that exhibited comparable fluorescence intensities. The results show that RUNX2 proteins with two different epitope tags are extensively colocalized in WC preparations (40% of cells: >90% signal overlap of red and green fluorescence; 60% of cells: 60-90% signal overlap) and NMIF preparations (30% of cells: >90% signal overlap; 70% of cells: 60-90% signal overlap). The extent of colocalization of RUNX1 and RUNX2 proteins is very similar to that observed for RUNX2 proteins carrying two distinct tags (compare Figures 4A and B); all cells in which both proteins were expressed displayed extensive or complete signal overlap. The colocalization of red and green fluorescence is observed by the yellow fluorescence in the merged images (Figures 4A and B). Since RUNX1 and RUNX2 proteins are widely distributed and some signal overlap may be expected due to random chance, we compared the localization of RUNX2 with another transcription factor, which is also widely distributed, Sp1 (Figure 4C). This result showed little or no signal overlap between RUNX2 and Sp1, suggesting that colocalization of RUNX1 and RUNX2 is specific. Taken together, these results demonstrate that RUNX1 and RUNX2 are targeted to common subnuclear domains, which reflects the amino acid sequence similarities of their targeting signals (Zeng et al., 1997; Zaidi et al., 2001). Our findings are consistent with the concept that RUNX1 and RUNX2 bind to a common acceptor protein at nuclear matrix-associated subnuclear sites.
EGFP-RUNX2 Colocalizes with the CBFβ Binding Partner

To assess whether EGFP-RUNX2 and the RUNX interacting partner protein CBFβ are targeted to common subnuclear domains, we transiently co-transfected SaOS-2 cells with EGFP-RUNX2 and CBFβ expression plasmids and isolated WC and NMIF preparations. CBFβ is present in the cytoplasm and the nucleus in whole cell (WC) preparations (Figure 5). In addition, CBFβ is observed as a nuclear protein in WC and NMIF preparations. Whether CBFβ is present in the cytoplasm and nucleus or only in the nucleus may depend on the ratio of EGFP-RUNX2 and CBFβ protein concentrations expressed (Figure 5; Kundu et al., 2002). CBFβ and EGFP-RUNX2 colocalize in common subnuclear domains as shown by the appearance of yellow foci in the merged images and the coincidence of red and green signal maxima in the line scans in the nucleus of WC and NMIF preparations (Figure 5). These results are consistent with the findings that RUNX2 and CBFβ physically interact by immunoprecipitation assays and colocalize in HeLa and ROS 17/2.8 cells using immunofluorescence microscopy (Kundu et al., 2002).

RUNX Domains Are Associated with Sites of Active Transcription

Since RUNX proteins and the CBFβ binding partner localize in common subnuclear domains and form a transcription factor complex, we evaluated whether transcription was occurring at these subnuclear foci. SaOS-2 cells were transfected with either EGFP-RUNX1 or EGFP-RUNX2. Nascent transcripts were labeled by incorporation of BrUTP into newly made transcripts and NMIF preparations were performed. The BrUTP labeled transcripts were detected by immunofluorescence
Figure 5. EGFP-RUNX2 and CBFβ Are Targeted to Common Subnuclear Domains. SaOS-2 cells were transiently transfected with 0.25 µg of EGFP-RUNX2 and CBFβ expression constructs and WC and NMIF preparations were performed. CBFβ was detected using a mouse monoclonal CBFβ antibody (Wang et al., 1996b; 1:20 dilution). EGFP-RUNX2 (green) is a nuclear protein whereas CBFβ (red) is localized to the cytoplasm and is translocated into the nucleus by association with RUNX proteins. WC images are shown for CBFβ localized to the cytoplasm and nucleus and only to the nucleus. Merged images show colocalization of EGFP-RUNX2 and CBFβ in the nucleus as yellow fluorescence. The white line in the merged images represents the area of the nucleus that the extent of colocalization was determined by the line scan function from MetaMorph. Line scans images show colocalization of EGFP-RUNX2 and CBFβ when the green and red peaks line up at the same nuclear position.
microscopy using a rat monoclonal α-BrdU antibody and an Alexa-568 α-rat secondary antibody. Sites of BrUTP fluorescence represent sites of active transcription. Using confocal microscopy we find that the majority of both the EGFP-RUNX1 and EGFP-RUNX2 foci colocalize with sites of BrUTP incorporation (Figure 6A and B). The extent of EGFP-RUNX1 colocalization with BrUTP was 73 ± 5% and BrUTP colocalization with EGFP-RUNX1 is 78 ± 5% (n=13). The extent of EGFP-RUNX2 colocalization with BrUTP was 83 ± 4% and BrUTP colocalization with EGFP-RUNX2 is 86 ± 2% (n=9). Matching red and green peaks in the same positions of the line scans (shown below images) demonstrate points of colocalization between EGFP-RUNX1 or EGFP-RUNX2 and BrUTP labeling. These results indicate that active transcription occurs at a significant subset of the punctate RUNX foci. These results taken together with previous findings, which show that several co-regulatory factors and nascent transcripts associate with RUNX subnuclear foci, suggest that the subnuclear foci may represent transcriptional multi-subunit regulatory complexes (Zaidi et al., 2002; Javed et al., 2000; Zeng et al., 1998; Kundu et al., 2002).

**Intranuclear Trafficking of RUNX2 in Living Cells Is Dependent on the C-terminal Domain**

To evaluate whether RUNX1 and RUNX2 are localized to punctate foci in living cells, we examined the subnuclear organization of EGFP-RUNX1 and EGFP-RUNX2 fusion proteins in SaOS-2 cells using time-lapse microscopy. For comparison, the subnuclear distribution of EGFP alone was analyzed. To assess
Figure 6. RUNX Proteins Localize to Sites of Active Transcription. SaOS-2 cells were transiently transfected with (A) EGFP-RUNX1 and (B) EGFP-RUNX2. Nascent transcripts were labeled with BrUTP for 30 minutes. Confocal microscopy was used to capture images of the intrinsic green fluorescence of EGFP and BrUTP labeling using a rat α-BrdU antibody (red). Merged images show in NMIF preparations colocalization of EGFP-RUNX1 and EGFP-RUNX2 with BrUTP incorporation (yellow) in a significant subset of foci. Images shown are 3-D projections (top) and a center section (bottom). The scale bars equal 10 μm. Line scans are shown below the images to show the extent of colocalization of red and green fluorescence along the white lines indicated in the merged 3D projections.
movement of the foci, we captured time-lapse images every 10-30 seconds for 20-30 minutes using exposure times of 100 or 200 milliseconds. Only those cells that exhibited significant signal above background were analyzed. The results show that cells expressing EGFP alone produce a diffuse fluorescence signal with comparable intensity in the cytoplasm and nucleus (Figure 7A; see supplementary CD for movies, Movie 1A). The movement of EGFP proteins is most clearly observed in the time-lapse video micrographs. In living cells expressing EGFP-RUNX1 or EGFP-RUNX2, we observe punctate domains in the nucleus (Figures 7B and 7C), which are very similar to those in fixed preparations (see Figure 2). Furthermore, these foci remain stationary within the nuclear space throughout the 30-minute time of observation (Figures 7B and 7C; Movies 1B and 1C). In the timelapse movies of cells expressing EGFP-RUNX1 and EGFP-RUNX2 the foci appear to move slightly. However, this apparent movement is limited relative to the diameter of the nucleus and may represent either changes in the shape of the foci and/or nuclei or movement of cells relative to the plane of focus. These results show that the punctate foci observed in fixed cells are bona fide subnuclear domains that can be visualized in living cells and that these punctate domains are relatively stable in the nucleus over time.

To determine whether the C-terminus of RUNX2 is necessary for the localization of RUNX proteins to punctate foci in living cells, we analyzed the RUNX2 deletion mutant lacking the C-terminus (EGFP-RUNX2Δ361). This fusion protein displays a diffuse fluorescence signal throughout the cytoplasm and nucleus (Figure 7D; Movie 1D), which is similar to EGFP alone (compare Figures 7A and
Figure 7. RUNX1 and RUNX2 Subnuclear Foci Are Stationary in Living Cells within the Nuclear Space. SaOS-2 cells were transiently transfected with (A) EGFP; (B) EGFP-RUNX1; (C) EGFP-RUNX2 and (D) EGFP-RUNX2Δ361 expression vectors. Time-lapse images show pictures captured at 0, 5, 10, 15 and 20 minutes (A) or 0, 5, 10, 20, and 30 minutes (B, C, and D) using 100 milliseconds (A and B) or 200 milliseconds (C and D) exposure times. Arrowheads (in B and C) illustrate examples of stationary RUNX subnuclear domains. RUNX proteins do not localize to the nucleoli (dark spots). Scale bars equal 10 μm.
A  EGFP  
0 min.  5 min.  10 min.  15 min.  20 min.

B  EGFP-RUNX1  
0 min.  5 min.  10 min.  20 min.  30 min.

C  EGFP-RUNX2  
0 min.  5 min.  10 min.  20 min.  30 min.

D  EGFP-RUNX2Δ361  
0 min.  5 min.  10 min.  20 min.  30 min.
7D; Movies 1A and 1D). However, the fluorescence intensity of EGFP-RUNX2Δ361 was greater in the nucleus than in the cytoplasm (Figure 7D). Moreover, the subnuclear distribution of EGFP-RUNX2Δ361 is very different from the punctate foci observed for wild-type RUNX1 and RUNX2. Thus, our results indicate that the C-terminus is required for localization of RUNX2 into punctate subnuclear domains.

**Deletion of the C-terminus of RUNX2 Increases the Intranuclear Mobility of RUNX2 Proteins**

The relative mobility of the EGFP-RUNX fusion proteins was determined by using Fluorescence Recovery After Photobleaching (FRAP) analysis. SaOS-2 cells were transiently transfected with EGFP-RUNX1, EGFP-RUNX2, EGFP-RUNX2Δ361 or EGFP alone. In FRAP, a defined area in the nucleus of cells expressing each of these proteins was photobleached with a laser beam for 100 ms and the recovery of the fluorescence was determined. Photobleaching represents the irreversible loss of EGFP fluorescence. The recovery of the fluorescence in the photobleached area represents the movement of unbleached molecules into the photobleached area. The recovery kinetics provide an indication of the association of the RUNX protein with other nuclear components. The half-time of recovery is the time on the recovery curve (fluorescence intensity vs. time) at half the maximal height of recovery and represents the speed at which the EGFP-RUNX proteins move. The immobile fraction is the percent of the initial fluorescence that is not regained after recovery and represents the percent of proteins that do not move in the time of the experiment.
Recovery of the fluorescence signal in the entire bleached area was determined by capturing sequential images following photobleaching and calculating the half-time of recovery and percent immobile fraction as described in the Chapter 1, Materials and Methods (Figure 8; Table 1). The estimated half-time of recovery of EGFP-RUNX1 and EGFP-RUNX2 proteins, respectively, is calculated to be 10.2 ± 0.6 (n=5) and 10.7 ± 1.1 (n=10) seconds and the mean percent immobile fraction is calculated to be 20.0 ± 2.8% and 26.1 ± 7.1% (Table 1). These results suggest that 75-80% of EGFP-RUNX1 and EGFP-RUNX2 proteins move very quickly through the nucleus and 20-26% are immobilized by interacting with the nuclear architecture. These findings were reproduced in independent experiments (n=15). We observed that the punctate foci containing EGFP-RUNX1 and EGFP-RUNX2 proteins recovered after photobleaching with a similar morphology as before photobleaching and in the same location (Figures 8A and 8B; see Movies 2A and 2B), indicating that EGFP-RUNX proteins are rapidly exchanging at subnuclear foci. Additionally, we analyzed the dynamic exchange of RUNX factors at specific foci in the photobleached area (boxed areas in Figures 8A and 8B show examples; Table 2) compared to the entire photobleached area. For EGFP-RUNX1 and EGFP-RUNX2 the mean half-time of recovery of the foci was similar to that of the entire photobleached area (Table 2). The mean percent immobile fraction for the foci is 29.7 ± 4.5% for EGFP-RUNX1 and 32.1 ± 7.9% for EGFP-RUNX2. The percent immobile fraction for the foci is slightly higher than that for the entire photobleached area, suggesting that EGFP-RUNX1 and EGFP-RUNX2 may be more tightly bound to the foci than the diffuse area around the foci, which may reflect association to the
Figure 8. RUNX1 and RUNX2 Dynamically Associate with Subnuclear Foci in Living Cells in a C-terminal Dependent Mechanism. SaOS-2 cells were transiently transfected with (A) EGFP-RUNX1; (B) EGFP-RUNX2; (C) EGFP-RUNX2Δ361 and (D) EGFP expression vectors. Pre-bleached images are shown. The circles represent the entire photobleached areas in which the recovery rates were calculated. The black boxes (in A and B) represent the area encompassing the foci within the photobleached area in which the recovery rates were also determined. Shown images were captured before photobleaching and 1, 3, 5, 10 and 45 seconds after photobleaching. The white bars correspond to a scale of 10 microns. (E) Recovery curves of the proteins are shown as relative fluorescence intensity vs. time. From these curves the half-time of recovery and percent immobile fraction were calculated as described in the Methods. The line at a relative intensity of 1.0 represents the fluorescence intensity before bleaching.
C  EGFP-RUNX2Δ361

Pre-bleach  1 s  3 s

5 s  10 s  45 s

D  EGFP

Pre-bleach  1 s  3 s

5 s  10 s  45 s
Pre-bleach

Relative Fluorescence Intensity

EGFP-RUNX1
EGFP-RUNX2
EGFPRUNX2Δ361
EGFP

time (s)
Table 1. Recovery of Entire Photobleached Area

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Half-time of recovery</th>
<th>Immobile fraction</th>
</tr>
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<tbody>
<tr>
<td>EGFP-RUNX1</td>
<td>10.2 ± 0.6 s</td>
<td>20.0 ± 2.8%</td>
</tr>
<tr>
<td>EGFP-RUNX2</td>
<td>10.7 ± 1.1 s</td>
<td>26.1 ± 7.1%</td>
</tr>
<tr>
<td>EGFP-RUNX2ΔC</td>
<td>&lt; 600 ms</td>
<td>0%</td>
</tr>
<tr>
<td>EGFP</td>
<td>&lt; 400 ms</td>
<td>0%</td>
</tr>
</tbody>
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Table 2. Recovery of RUNX Foci in the Photobleached Area

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Half-time of recovery</th>
<th>Immobile fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-RUNX1</td>
<td>6.7 ± 0.2 s</td>
<td>29.7 ± 4.5%</td>
</tr>
<tr>
<td>EGFP-RUNX2</td>
<td>6.1 ± 1.0 s</td>
<td>32.1 ± 7.9%</td>
</tr>
</tbody>
</table>
nuclear matrix and/or co-regulatory proteins at subnuclear foci. Thus, these results indicate that RUNX proteins undergo dynamic exchange at the stationary subnuclear punctate domains.

We then assessed the effect of deleting the C-terminus on the relative mobility of RUNX2. RUNX2 proteins that have the C-terminus deleted (EGFP-RUNX2Δ361) exhibit a mobility comparable to that of EGFP alone. EGFP and EGFP-RUNX2Δ361 proteins (Figures 8C and 8D; see Movies 2C and 2D) are completely mobile, since the percent immobile fraction is zero (Table 1). The relative recovery curves of EGFP-RUNX1, EGFP-RUNX2, EGFP-RUNX2Δ361 and EGFP proteins are shown in Figure 8E and represent the initial recovery curves of the entire photobleached area before correcting for the extent of photobleaching of the entire nucleus. The estimated half-time of recovery is <600 milliseconds for EGFP-RUNX2Δ361 and <400 milliseconds for EGFP alone (Table 1). Both EGFP-RUNX2Δ361 and EGFP are almost completely recovered within 1 second after photobleaching (Figures 8C and 8D; Table 1), indicating that EGFP-RUNX2Δ361 and EGFP are highly mobile. The increased mobility of RUNX2Δ361 compared to full length proteins suggests that deletion of the C-terminal domain perturbs the association of RUNX proteins with subnuclear foci in living cells. One plausible interpretation of our finding is that the C-terminus together with its interacting proteins contributes to the stabilization of RUNX subnuclear foci. Another interpretation is that the mobility of RUNX factors may be retained at subnuclear foci by the interaction of the C-terminus with the nuclear matrix and/or co-regulatory proteins.
DISCUSSION

In this study, RUNX transcription factors were shown to associate dynamically with stationary subnuclear foci in living cells. Furthermore, the C-terminal segment of RUNX factors, which contains the nuclear matrix targeting signal (NMTS), regulates intranuclear mobility by increasing the association of RUNX factors at their subnuclear foci in living cells. Both RUNX1 and RUNX2 proteins, which support development of different tissues and specification of distinct cell types, localize to the same foci when co-expressed exogenously in the same cell. This result suggests that homologous targeting signals present in the C-termini of RUNX proteins (Zeng et al., 1997; Zaidi et al., 2001) may direct RUNX factors to common subnuclear domains. Moreover, our findings together with previous results show that RUNX subnuclear foci contain co-regulatory factors and nascent transcripts, suggesting that these subnuclear foci may represent multi-subunit complexes to regulate transcription (Zaidi et al., 2002; Javed et al., 2000; Zeng et al., 1998; Kundu et al., 2002).

Multi-subunit transcription complexes appear to be dynamically assembled from RUNX proteins and co-regulatory factors at subnuclear foci. This is supported by the findings that a subset of RUNX foci colocalize with sites of active transcription (Chapter 1, Figure 6) and that RUNX interacting proteins such as CBFβ are also targeted to RUNX foci (Chapter 1, Figure 5; Zaidi et al., 2002; Kundu et al., 2002). CBFβ interacts with RUNX proteins, but does not bind directly to DNA, and increases RUNX-mediated transcription activity (Speck and Stacy, 1995; Wang et al.,...
1993; Ogawa et al., 1993a; Harada et al., 1999; Kundu et al., 2002). Furthermore, several co-regulatory proteins, including TLE, Smad and YAP have been shown to colocalize with RUNX subnuclear foci (Javed et al., 2000; Zaidi et al., 2002; unpublished data from S. K. Zaidi). Taken together, these results suggest that subnuclear foci may represent sites of transcription containing multi-subunit transcription factor complexes.

The C-terminal truncation of RUNX1 and RUNX2, which removes the intranuclear targeting signal, results in a lethal phenotype in vivo, suggesting that the C-termini of RUNX proteins are essential for functional activity (North et al., 1999; Choi et al., 2001). The C-terminal segment of RUNX proteins appears to reduce the mobility of these proteins by mediating association with nuclear architecture, perhaps by supporting in situ formation of complexes. Interactions of the C-termini of RUNX factors with co-repressors and co-activators are important for regulation of transcription (Hanai et al., 1999; Javed et al., 2000; Lutterbach and Hiebert, 2000). Our results presented here together with previous data suggest that RUNX proteins assemble into macromolecular complexes with co-regulatory proteins at nuclear matrix associated sites to regulate gene transcription (Berezney and Wei, 1998; Lutterbach and Hiebert, 2000; Stein et al., 2000a; Javed et al., 2000; Zeng et al., 1997; Zeng et al., 1998; Zaidi et al., 2001). We propose that the functional activity of RUNX proteins at subnuclear foci may critically depend on the spatial-temporal availability of co-factors.

Foci that contain RUNX transcription factors remain stationary within the nuclear space, but are dynamic structures with which RUNX proteins continuously
associate and disassociate. Our results suggest that immobilization of these subnuclear domains within the nuclear space may reflect association with the nuclear matrix. Previous commentaries have argued that some subnuclear structures could be artifacts resulting from the fixation and/or extraction procedures (Pederson, 2000), as opposed to functional compartments that support gene expression (Penman, 1995; Stein et al., 2000b; Wei et al., 1998; Cook, 1999; Stenoien et al., 2000b; Nickerson, 2001). Here, we show that these RUNX transcription factor domains are observed in both fixed and living cells and that a subset of these foci represent active sites of transcription (as revealed by BrUTP labeling). Moreover, subnuclear foci in cells overexpressing RUNX proteins are comparable to subnuclear foci with endogenous RUNX proteins in that they are both punctate and nuclear matrix associated (Chapter 1, Figure 3; Prince et al., 2001). Thus, our findings suggest that the RUNX domains are functional subnuclear structures.

Our observation that RUNX proteins continuously and rapidly shuttle into and out of the dynamic, yet spatially stable foci may reflect a mechanism for the organization and reversible formation of transcriptional complexes in situ. However, the high percent immobile fraction obtained for RUNX1 and RUNX2 (20-26%), suggests that a fraction of RUNX proteins is immobilized by association with the nuclear architecture in a C-terminal dependent mechanism. The mobility of RUNX transcription factors into the stationary subnuclear domains occurs within the same time scale as the mobility of the alternative splicing factor (ASF) fused to green fluorescent protein (GFP) into splicing factor domains (Phair and Misteli, 2000; Kruhlak et al., 2000). These recent studies show that ASF-GFP rapidly associates
with splicing compartments and its mobility is 100 times slower than GFP alone (Phair and Misteli, 2000; Kruhlak et al., 2000). It has been well established that processing of gene transcripts occurs within specific domains (SC-35 “speckles”) in the nucleus, which reflects the spatial compartmentalization of the splicing machinery (Xing et al., 1993; Spector, 1993; Phair and Misteli, 2000; Kruhlak et al., 2000). Our data indicate that at least some tissue-specific transcription factors are similarly compartmentalized within the nucleus in living cells. Other nuclear proteins, such as GFP-Histone H2B (Kanda et al., 1998; Phair and Misteli, 2000) and GFP-lamin B receptor (Ellenberg et al., 1997) are extremely immobile in the nucleus and are less mobile that RUNX proteins. Taken together, these results suggest that attachment to the nuclear architecture immobilizes nuclear proteins.

Thus, we conclude that the dynamic association of RUNX factors to stationary subnuclear foci through a common C-terminal signal provides a biological mechanism for the formation of essential tissue related and gene specific regulatory complexes.
CHAPTER 2

Effect of the Nuclear Matrix Targeting Signal on the Subnuclear Distribution and Mobility of RUNX2
INTRODUCTION

A defined 31 to 38 amino acids nuclear matrix targeting signal (NMTS) located in the C-terminus of RUNX proteins is required for association of RUNX transcription factors with the nuclear matrix (Tang et al., 1999; Zeng et al., 1997; Zaidi et al., 2001). The NMTS was characterized in RUNX1 and RUNX2 by biochemical fractionation and in situ immunofluorescence assays using a series of deletion mutants (Zeng et al., 1997; Zaidi et al., 2001). The NMTS sequence is homologous in all three RUNX proteins (Zeng et al., 1997). Nuclear matrix targeting signals have been characterized in other proteins including, the glucocorticoid receptor (GR), Pit-1, YY1 and ETO, however these sequences are not similar to the RUNX NMTS (Tang et al., 1998; Mancini et al., 1999; Barseguian et al., 2002; McNeil et al., 1998). The crystal structure of the RUNX1 NMTS has a loop-turn-loop structure, suggesting that the two finger-like loops may associate with the nuclear matrix (Tang et al., 1999). In addition to the NMTS, the C-terminus of RUNX proteins contains several interaction sites for co-regulatory proteins. Specific combinations of these co-regulatory factors synergize with RUNX proteins to activate or repress specific genes (Lutterbach and Hiebert, 2000; Westendorf and Hiebert, 1999; Ito, 1999).

Several results in Chapter 1 lead to the question of whether the NMTS has an effect on the intranuclear mobility and subnuclear distribution of RUNX2. First, we demonstrated that RUNX subnuclear foci co-localize with sites of active transcription. Second, deletion of the C-terminus of RUNX2 (EGFP-RUNX2Δ361),
which removes the NMTS and several co-regulatory factor interaction domains created several effects on the transactivation potential, subnuclear distribution and mobility of RUNX2. Specifically, the transactivation potential of the osteocalcin promoter by EGFP-RUNX2Δ361 is reduced three-times compared to wild-type EGFP-RUNX2. The subnuclear punctate foci are no longer observed after deletion of the C-terminus. EGFP-RUNX2Δ361 is not associated with the nuclear matrix. Finally, the intranuclear mobility of EGFP-RUNX2Δ361 is increased 25-fold compared to wild-type EGFP-RUNX2. Taken together, these results suggest that interaction of the C-terminus with the nuclear matrix and/or co-regulatory proteins reduce the intranuclear mobility of RUNX proteins to regulate gene expression at subnuclear foci. However, the region of the deleted C-terminus responsible for the interaction that decreases the mobility of RUNX2 is unknown. Therefore, we hypothesized that the RUNX NMTS may reduce the mobility of RUNX proteins through association with the nuclear matrix to support transcriptional control.

To determine whether the NMTS influences the mobility of RUNX proteins, we generated two proteins with single point mutations in the NMTS of RUNX2 (R383A and Y413A in the Type I isoform). Fluorescence microscopy, time-lapse microscopy and fluorescence recovery after photobleaching (FRAP) analysis were used to determine their subnuclear distribution and mobility compared to wild-type RUNX2. The results show that the RUNX2 NMTS point mutant proteins (i.e., R383A and Y413A) have a decreased ability to associate with the nuclear matrix in comparison to wild-type RUNX2. However, the NMTS point mutations have no measurable effect on the intranuclear mobility of RUNX2 compared to wild-type
protein. Moreover, the subnuclear distribution of the NMTS mutants is similar to wild-type RUNX2. These results show that the mobility and subnuclear distribution of the RUNX2 NMTS point mutants R383A and Y413A are similar to those of wild-type RUNX2. Hence, the single amino acid substitutions at amino acids 383 and 413 are ineffective and do not change the mobility of RUNX2. Rather, the mobility may be influenced by the association of co-regulatory proteins that bind to the C-terminus independent of the NMTS point mutations. Alternatively, the entire NMTS may be required to decrease the mobility of RUNX2.
MATERIALS AND METHODS

Cell Culture

Human osteosarcoma SaOS-2 cells (American Type Culture Collection (ATCC), Manassas, VA) were maintained in McCoy's 5A medium supplemented with 15% (v/v) fetal bovine serum (FBS), 100 Units/ml Penicillin, 100 μg/ml Streptomycin and 2 mM L-glutamine at 37 °C in a humidified 5% CO₂ incubator. Medium was changed every 2-3 days.

Plasmids

HA-RUNX2 R398A and HA-RUNX2 Y428A contain mutations in the NMTS of the til-I (Type II) isoform (528 amino acids) of RUNX2 at amino acids R398 and Y428 (Zaidi et al., 2002). Because the Type II isoform has a 15 amino acid extension on its N-terminus compared to PEBP2αA (Type I isoform), these mutations are equivalent to the mutations R383A and Y413A in the Type I isoform of RUNX2 (513 amino acids), which is cloned into EGFP-RUNX2 (Figure 1). The C-terminals of the Type I and Type II isoforms are exactly the same. Therefore, we replaced the C-terminus of EGFP-RUNX2 with the C-terminals of RUNX2 containing either of the two NMTS mutations to generate the constructs EGFP-RUNX2 R383A and EGFP-RUNX2 Y413A. Briefly, plasmid DNAs encoding HA-RUNX2 R398A, HA-RUNX2 Y428A and EGFP-RUNX2 were digested with BstEII and EcoRI. The 1 kb C-terminal BstEII/EcoRI fragment for each of the NMTS mutations was isolated and ligated to the 5.7 kb EGFP-RUNX2 BstEII/EcoRI fragment, creating EGFP-RUNX2
Figure 1. Generation of EGFP-RUNX2 NMTS Mutants. The HA-RUNX2 NMTS mutant constructs contained the tilt-1 (Type II) isoform of RUNX2 (528 amino acids) and the EGFP-RUNX2 construct contained the PEBP2αA (Type I) isoform of RUNX2 (513 amino acids). HA-RUNX2 R398A and Y428A and the EGFP-RUNX2 constructs were digested with BstEII and EcoRI. The C-terminus of EGFP-RUNX2 (BstEII/EcoRI fragment) was replaced with the BstEII/EcoRI fragment containing the R398A and Y428A creating EGFP-RUNX2 R383A and Y413A NMTS mutants. The difference in numbers are created because of the 15 amino acid extension in the N-terminus of the Type II isoform compared to the Type I isoform. Dotted lines (-----) show equivalent amino acids in the Type I and Type II isoforms. Broken lines (----) represent BstEII and EcoRI restriction sites.
R383A and EGFP-RUNX2 Y413A. Both clones were sequenced using the automated ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA).

**Western Blot Analysis**

SaOS-2 cells were plated at a density of 1.0-1.5 x 10^6 cells in 100 mm plates and transfected with 4 μg of expression plasmid, 10 μl of Plus reagent and 15 μl of Lipofectamine reagent (Invitrogen, Carlsbad, CA). Cell pellets were collected and lysed as described in Chapter 1. For each sample 20 μg of total proteins were separated on a 10% SDS-PAGE gel. EGFP proteins were detected using a mouse monoclonal GFP antibody (Clontech; 1:10,000 dilution). HA-tagged proteins were detected using a mouse monoclonal HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5,000 dilution). RUNX proteins were detected with a rabbit polyclonal antibody to RUNX2 (Ab-1; Oncogene, Boston, MA; 1:10,000 dilution). Appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; 1:10,000 dilution) were detected using the Western Lightning chemiluminescence kit (NEN, Boston, MA). Cdk2 protein was detected using a rabbit polyclonal α-cdk2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5,000 dilution) as a control for protein loading.

**In situ Immunofluorescence**

SaOS-2 cells were grown on 0.5% (w/v) gelatin-coated coverslips and cultured to 70% confluence. Cells were transiently transfected with 0.5 μg of expression plasmid, 3 μl Plus reagent and 2.5 μl Lipofectamine reagent (Invitrogen,
Carlsbad, CA). Cells were harvested 18-20 hours post transfection. Whole cell (WC), cytoskeleton (CSK) and nuclear matrix-intermediate filament (NMIF) preparations were performed as described in Chapter 1. Cells were counted for retention of RUNX proteins in the NMIF preparations in two experiments.

**Time-Lapse Imaging**

SaOS-2 cells were plated at a cell density of $1.0 \times 10^6$ in 100 mm plates containing gelatin-coated 40 mm coverslips (Bioptechs, Butler, PA). Cells were then transiently transfected using 1-4 μg of either EGFP-RUNX2, EGFP-RUNX2 R383A, or EGFP-RUNX2 Y413A expression plasmids, 10 μl Lipofectamine reagent and 15 μl Plus reagent (Invitrogen, Carlsbad, CA). Coverslips were assembled into the FCS-2 closed cell chamber (Bioptechs, Butler, PA) in which a peristaltic pump (Instech Laboratories Inc, Plymouth Meeting, PA) was used to perfuse complete McCoy’s 5A media without phenol red (US Biological, Cleveland, OH) and 10 nM Mitotracker Red dye through the chamber. Cells were maintained at 37°C and time-lapse images were captured every 20-30 seconds for 20-30 minutes and processed as described in Chapter 1. Exposure times for EGFP fusion proteins were 100-200 milliseconds. The number of cells with each of the three different subcellular distributions, categorized as punctate, punctate/diffuse and diffuse cells, were counted.

Significance between the wild-type and NMTS mutants and between the subcellular distributions were determined using the analysis of variance (ANOVA) and multiple pair-wise comparisons in the Tukey’s studentized range test and Bonferroni t-test.
**Fluorescence Recovery After Photobleaching (FRAP) Analysis**

SaOS-2 cells were plated in T-25 flasks at a density of $1.0 \times 10^6$ cells/flask and cultured until 70% confluency. Expression plasmids (2 μg) were transiently transfected using 5 μl of Lipofectamine and 4 μl Plus reagents (Invitrogen, Carlsbad, CA). Transfected cells were and plated in coverslip live cell chambers and FRAP analysis was performed as in Chapter 1 except that images of fluorescence recovery were captured every second for 30-60 seconds using 200 millisecond exposure times. The half-time of recovery ($t_{1/2}$) and the percent immobile fraction were calculated as described in Chapter 1. The recovery rates were calculated for the entire photobleached area. Adobe Photoshop and Microsoft PowerPoint were used to assemble the digital images. Standard errors were determined as the standard error of the mean (SEM). Significance between wild-type RUNX2 and the NMTS mutants and between the subnuclear distributions was determined using the analysis of variance (ANOVA) and multiple pair-wise comparisons in the Tukey's studentized range test and Bonferroni t-test.

**Movies on Supplementary CD**

QuickTime Movies 4A-C and 5A-C (available on the Supplementary CD) show the time-lapse images corresponding to Figures 4A-C and 5A-C. Time-lapse images were captured using 200 ms exposure times Movie 4A, every 30 seconds for 30 minutes; Movie 4B, every 30 seconds for 30 minutes; Movie 4C, every 20 seconds for 30 minutes; Movie 5A, every 30 seconds for 30 minutes; Movie 5B, every 20 seconds for 20 minutes; and Movie 5C, every 20 seconds for 20 minutes. QuickTime
Movies 6A-C, 7A-B, 8A-C show cells corresponding to those captured in Figure 6A-C, 7A-B, 8A-C for FRAP analysis. Movies 6A-C, 7A-B, 8A-B show sequential images captured before bleaching and every second for 60 seconds after photobleaching. Movie 8C shows sequential images captured before bleaching and every second for 30 seconds after photobleaching.
RESULTS

Generation of EGFP-RUNX2 Nuclear Matrix Targeting Signal Mutant Proteins

We have shown that deletion of the C-terminus, which removed the nuclear matrix targeting signal (NMTS) and several co-regulatory proteins interaction domains, increased the mobility of RUNX2 (see Chapter 1). Therefore, we determined whether the NMTS by itself has an effect on the mobility of RUNX2. We created R383A and Y413A mutations in the NMTS of EGFP-RUNX2 (Figure 1) based on the RUNX2 NMTS point mutations that were previously demonstrated to decrease nuclear matrix association and transactivation of the osteocalcin promoter compared to wild-type RUNX proteins (Zaidi et al., 2002; unpublished results). The R383A mutation in EGFP-RUNX2 is located N-terminal to the RUNX2 NMTS, whereas the Y413A point mutation in EGFP-RUNX2 is located in Loop II of the NMTS crystal structure (Figure 1) (Tang et al., 1999).

To determine whether the EGFP-RUNX2 NMTS mutant proteins were expressed, western blot analysis was performed using SaOS-2 whole cell extracts transiently transfected with wild-type EGFP-RUNX2, EGFP-RUNX2 R383A and EGFP-RUNX2 Y413A (Figure 2). Both GFP (Figure 2A) and RUNX2 (Figure 2B) antibodies were used to detect the EGFP-RUNX fusion constructs and an HA antibody (Figure 2A) was used to detect HA-RUNX2 R398A and Y428A (Type II isoform). EGFP-RUNX2Δ361 and EGFP were used as controls for expression. An α-CDK2 antibody was used as a control for protein loading (Figure 2, bottom panels). The results show that the EGFP-RUNX2 NMTS mutants have the same molecular mass and are expressed at comparable levels as the wild-type EGFP-RUNX2 protein.
Figure 2. EGFP-RUNX2 NMTS Mutants Are Expressed at Similar Levels as Wild-Type EGFP-RUNX2. Wild-type and mutant EGFP-RUNX fusion proteins as indicated above each lane were transfected in SaOS-2 cells and whole cell extracts were subjected to western blot analysis. (A) A monoclonal mouse GFP antibody was used to detect the EGFP-RUNX fusion proteins and an HA antibody was used to detect the HA-RUNX2 NMTS mutants. Arrows indicate expressed proteins, which are labeled above the lanes. (B) A rabbit polyclonal RUNX2 antibody was used to detect RUNX2 protein. Endogenous RUNX2 is labeled with the lower arrow. The upper arrows indicate EGFP- and HA-tagged RUNX fusion proteins. (C) A rabbit polyclonal RUNX1 antibody was used to detect EGFP-RUNX1, overexpressed Untagged-RUNX1 and endogenous RUNX1 protein (arrows). (A, B, C) Cdk2 antibody was used as a control for protein loading.
Furthermore, the expression levels of EGFP-RUNX2 and EGFP-RUNX2Δ361 were equivalent in SaOS-2 cells (Figure 2), which were different than in HeLa cells (see Chapter 1, Figure 1) where the expression level of EGFP-RUNX2Δ361 was higher than that of EGFP-RUNX2.

To assess whether the EGFP-RUNX2 NMTS mutants are associated with the nuclear matrix, wild type EGFP-RUNX2 and mutant proteins EGFP-RUNX2 R383A (Figure 3A) and EGFP-RUNX2 Y413A (Figure 3B) were expressed in SaOS-2 cells and whole cell (WC), cytoskeleton (CSK) and nuclear matrix-intermediate filament (NMIF) preparations were performed. The results show that the NMTS mutants have a punctate subnuclear distribution and are associated with the nuclear matrix (Figure 3A and B). However, the percentage of cells with wild-type and NMTS mutant RUNX2 proteins retained in the nuclear matrix was determined by counting cells using fluorescence microscopy. The results demonstrate that the percent of cells in which RUNX proteins are retained in the nuclear matrix is less for the NMTS mutant proteins compared to wild-type RUNX2 proteins (Table 1). In 95% of cells expressing wild-type EGFP-RUNX2, EGFP-RUNX2 is associated with the nuclear matrix (NMIF/WC ratio, Table 1). However, in 27% of cells expressing EGFP-RUNX2 Y413A and in 60% of cells expressing EGFP-RUNX2 R383A, the NMTS mutant proteins are associated with the nuclear matrix. The percent of cells scored as positive (associated with the nuclear matrix) may decrease because overall, there may be a decrease affinity of the nuclear matrix associated proteins in each cell such that a substantial fraction fall below threshold. These results demonstrate that the NMTS point mutations decrease, but do not abolish, nuclear matrix association of RUNX2.
Figure 3. EGFP-RUNX2 NMTS Mutants Are Retained in the Nuclear Matrix.

SaOS-2 cells were transiently transfected with (A) EGFP-RUNX2 R383A or (B) EGFP-RUNX2 Y413A. Whole cell (WC), cytoskeleton (CSK) and nuclear matrix-intermediate filament (NMIF) preparations were performed. (A and B) Left panels show EGFP fluorescence images captured using a FITC filter. Cells were stained with DAPI (0.05 μg/ml) (right panels). Chromatin digested NMIF preparations show no DAPI staining.
Table 1. Counts of Nuclear Matrix Retention of Wild-type EGFP-RUNX2 and the NMTS Mutants in Fixed SaOS-2 Cells

<table>
<thead>
<tr>
<th>Proteins</th>
<th># Positive Cells</th>
<th>Total # Cells</th>
<th>NMIF/WC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EGFP-RUNX2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>110 (21%)</td>
<td>514</td>
<td>0.95</td>
</tr>
<tr>
<td>NMIF</td>
<td>91 (20%)</td>
<td>461</td>
<td></td>
</tr>
<tr>
<td><strong>EGFP-RUNX2 R383A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>58 (17%)</td>
<td>352</td>
<td>0.60</td>
</tr>
<tr>
<td>CSK</td>
<td>29 (9%)</td>
<td>308</td>
<td></td>
</tr>
<tr>
<td>NMIF</td>
<td>25 (10%)</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td><strong>EGFP-RUNX2 Y413A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>47 (21%)</td>
<td>223</td>
<td>0.27</td>
</tr>
<tr>
<td>CSK</td>
<td>39 (9%)</td>
<td>444</td>
<td></td>
</tr>
<tr>
<td>NMIF</td>
<td>17 (6%)</td>
<td>298</td>
<td></td>
</tr>
</tbody>
</table>
and that the Y413A mutation is more effective in decreasing nuclear matrix association than the R383A mutation.

The Subnuclear Distribution of RUNX2 Nuclear Matrix Targeting Signal Mutants Is Similar to Wild-type RUNX2 in Living Cells

To evaluate the mobility of the foci and the subnuclear distribution of the EGFP-RUNX2 NMTS mutant proteins in living cells compared to wild-type EGFP-RUNX2, SaOS-2 cells were transiently transfected with EGFP-RUNX2, EGFP-RUNX2 R383A and EGFP-RUNX2 Y413A constructs and time-lapse microscopy was used to observe the distributions. Images were captured every 20-30 seconds for 20-30 minutes to determine the movement of the subnuclear foci in cells expressing the NMTS mutants (Figure 4-5A; see Supplementary CD for Movies 4-5A). The results demonstrate that the RUNX2 NMTS mutant foci are stationary (Figure 4-5A; Movie 4-5A), which is similar to wild-type RUNX2. Moreover, three different subnuclear distributions were observed for the RUNX2 NMTS mutants, which were designated punctate, punctate/diffuse and diffuse (Figures 4 and 5A-C and see Movies 4 and 5A-C). The differences in the subnuclear distribution were initially thought to be an effect of the NMTS mutations. However, after examination of cells expressing wild-type EGFP-RUNX2, the three distributions were also observed for wild-type RUNX2 in living cells. The distinctions between subnuclear distributions were made by observing living cells in the fluorescence microscope. Thus, the assignment to each category was subjective. The criteria for distinguishing between the different subnuclear distributions were the following: 1) the punctate distribution
Figure 4. EGFP-RUNX2 R383A NMTS Mutant Expressing Cells Show Three Subnuclear Distribution Patterns in Living Cells. SaOS-2 cells were transiently transfected with EGFP-RUNX2 R383A NMTS mutant and time-lapse microscopy was performed in living cells. Time-lapse images show three different subnuclear distributions (A) punctate (B) punctate/diffuse and (C) diffuse. (A, B, C) Images are shown for 0, 5, 10, 20 and 30 minutes.
EGFP-RUNX2 R383A

A. Punctate

0 min.  5 min.  10 min.  20 min.  30 min.

B. Punctate/Diffuse

0 min.  5 min.  10 min.  20 min.  30 min.

C. Diffuse

0 min.  5 min.  10 min.  20 min.  30 min.
Figure 5. EGFP-RUNX2 Y413A NMTS Mutant Expressing Cells Also Show Three Subnuclear Distribution Patterns in Living Cells. SaOS-2 cells were transiently transfected with EGFP-RUNX2 Y413A NMTS mutant and time-lapse microscopy was performed in living cells. Time-lapse images show three different subnuclear distributions (A) punctate (B) punctate/diffuse and (C) diffuse. Images are shown for (A) 0, 5, 10, 20 and 30 minutes and (B, C) 0, 5, 10, 15 and 20 minutes.
A. Punctate

B. Punctate/Diffuse

C. Diffuse
contained a great number of bright punctate subnuclear foci and very little diffuse staining (Figure 4A and 5A); 2) the diffuse pattern contained mostly diffuse fluorescence signal in the nucleus and 0-2 subnuclear foci (Figure 4C and 5C); and 3) the punctate/diffuse distribution was intermediate between the first two types and contained several subnuclear foci surrounded by diffuse fluorescence signal (Figure 4B and 5B). The percentage of cells expressing the wild type and mutant proteins in each of the subnuclear distribution patterns were counted in living cells (Table 2). The results show that there are no significant differences between the wild-type and NMTS mutant proteins (using multiple pair-wise comparisons in the Tukey’s studentized range test and Bonferroni t-test $p > 0.05$). Furthermore, there is no statistical difference between each of the subnuclear distributions in living cells (Table 2; $p > 0.05$). These results suggest that the NMTS point mutations R383A and Y413A do not effect the overall subnuclear distribution of RUNX2.

**Point Mutations in the Nuclear Matrix Targeting Signal Have No Measurable Effect on RUNX2 Intranuclear Mobility**

Deletion of the RUNX2 C-terminus increases the mobility of RUNX2 (see Chapter 1), suggesting that the mobility of RUNX2 is constrained by association of the C-terminus with the nuclear matrix and/or co-regulatory proteins. We hypothesize that nuclear matrix association may be responsible for reducing RUNX mobility. Therefore, we examined the mobility of the EGFP-RUNX2 NMTS point mutants compared to wild-type EGFP-RUNX2 using FRAP analysis. Wild-type and mutant proteins were expressed in SaOS-2 cells by transfecting 2 μg of the expression
### Table 2. Counts of Subnuclear Distributions in Living SaOS-2 Cells

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Subcellular Distribution</th>
<th>Average % expressing cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EGFP-RUNX2</strong> (n=257)</td>
<td>punctate</td>
<td>48 ± 6%</td>
</tr>
<tr>
<td></td>
<td>punc/diff</td>
<td>27 ± 11%</td>
</tr>
<tr>
<td></td>
<td>diffuse</td>
<td>25 ± 4%</td>
</tr>
<tr>
<td><strong>EGFP-RUNX2 R383A</strong> (n=200)</td>
<td>punctate</td>
<td>31 ± 1%</td>
</tr>
<tr>
<td></td>
<td>punc/diff</td>
<td>42 ± 2%</td>
</tr>
<tr>
<td></td>
<td>diffuse</td>
<td>28 ± 3%</td>
</tr>
<tr>
<td><strong>EGFP-RUNX2 Y413A</strong> (n=196)</td>
<td>punctate</td>
<td>30 ± 4%</td>
</tr>
<tr>
<td></td>
<td>punc/diff</td>
<td>32 ± 12%</td>
</tr>
<tr>
<td></td>
<td>diffuse</td>
<td>38 ± 16%</td>
</tr>
</tbody>
</table>

*No significant difference based on multiple pair-wise comparisons between wild-type and the NMTS mutants and between the subnuclear distributions using Tukey’s studentized range test and Bonferroni t-test*
constructs. Photobleaching experiments were performed as described in the Materials and Methods and in Chapter 1. The methods for calculating the half-time of recovery for the punctate and diffuse distributions were slightly different. The half-time of recovery of the NMTS mutants with a punctate subnuclear distribution was calculated with the same formula as for wild-type EGFP-RUNX2, whereas that of the NMTS mutants with a diffuse subnuclear distribution was calculated with the same formula as for EGFP-RUNX2Δ361. The two formulas shown in the Materials and Methods of Chapter 1 were derived from the same original formula however, for the punctate distribution the constant (t<sub>c</sub>) was derived from the slope of the graph (ln i<sub>∞</sub>-i<sub>t</sub> vs. time), which was determined from the fluorescence intensities inside the photobleached area, and for the diffuse distributions t<sub>c</sub> was calculated using a comparison of the fluorescence intensities inside the photobleached area to a region outside the photobleached area.

The reason for using two different formulas was that the recovery of the proteins with a diffuse distribution was extremely fast and the CCD camera speed was not as high as the movement of the proteins. Therefore, when the half-time of recovery of the proteins in the diffuse distribution was calculated by the same formula as for the punctate distribution, the half-time of the proteins in the diffuse distribution was no different than that of the punctate distribution, which did not appear to be true from comparing the images of the recovery of the proteins (see Figures 6 and 8). Furthermore, for the diffuse distribution it was easy to find an area outside the photobleached area that had a similar intensity as inside the photobleached area since the fluorescence intensity of the proteins in the diffuse distribution was fairly uniform.
over the nucleus. However, in the punctate cells, it was very difficult to find an area outside the photobleached area that was a similar intensity as inside the photobleached area because the intensity was different in every area of the nucleus and the signal recovered much slower than diffuse signal. Therefore, different ways of calculating the half-time of recovery for the punctate and diffuse subnuclear distributions using two formulas derived from the same original formula were necessary. Furthermore, the half-time of recovery for the proteins with a punctate/diffuse subnuclear distribution could not be determined since the calculated half-time of recovery using the formula for the punctate distribution did not appear to reflect the recovery in the images. In addition, it was also difficult to find an area outside the photobleached area that was a similar intensity as inside the photobleached area using the formula for the diffuse subnuclear distribution.

Images of the recovery of wild-type and NMTS mutant EGFP-RUNX proteins, shown for the punctate (Figure 6), punctate/diffuse (Figure 7) and diffuse (Figure 8) distributions, demonstrate that the diffuse fluorescence recovers faster than the punctate fluorescence (see Supplementary CD; Movies 6A-C, 7A-B, 8A-C). Only cells with a punctate subnuclear distribution were used for calculations of the recovery rates of wild-type EGFP-RUNX2 protein, since at the time the FRAP experiments were performed it was not established yet that the wild-type protein also had the three types of subnuclear distributions in living cells. However, for the EGFP-RUNX2 NMTS mutants, the half-time of recovery and percent immobile fraction were calculated for each of the types of subnuclear distributions. The results show that wild-type EGFP-RUNX2 proteins expressed in SaOS-2 cells had an
Figure 6. Punctate Subnuclear Distribution Recover Slower than in the Other Subnuclear Distributions. SaOS-2 cells were transiently transfected with (A) EGFP-RUNX2, (B) EGFP-RUNX2 R383A or (C) EGFP-RUNX2 Y413A. Images of cells with a punctate subnuclear distribution are shown. Cells were replated in coverslip chambers for FRAP analysis. Pre-bleached images are shown. The circles represent the entire photobleached areas in which the recovery rates were calculated. Shown images were captured before photobleaching and 1, 3, 5, 10 and 60 seconds after photobleaching. The white bars correspond to a scale of 10 microns.
A  EGFP-RUNX2

<table>
<thead>
<tr>
<th></th>
<th>Pre-bleach</th>
<th>1 s</th>
<th>3 s</th>
<th>5 s</th>
<th>10 s</th>
<th>60 s</th>
</tr>
</thead>
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B  EGFP-RUNX2 R383A

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<tr>
<th></th>
<th>Pre-bleach</th>
<th>1 s</th>
<th>3 s</th>
<th>5 s</th>
<th>10 s</th>
<th>60 s</th>
</tr>
</thead>
</table>
C  EGFP-RUNX2 Y413A

Pre-bleach  1 s  3 s
5 s  10 s  60 s
Figure 7. FRAP Analysis of Punctate/Diffuse Subnuclear Distribution. SaOS-2 cells were transiently transfected with either (A) EGFP-RUNX2 R383A or (B) EGFP-RUNX2 Y413A and images for cells with a punctate/diffuse subnuclear distribution are shown. Cells were replated in coverslip chambers for FRAP analysis. Pre-bleached images are shown. The circles represent the entire photobleached areas in which the recovery rates were calculated. Shown images were captured before photobleaching and 1, 3, 5, 10 and 60 seconds after photobleaching. The white bars correspond to a scale of 10 microns.
A  EGFP-RUNX2 R383A

Pre-bleach  1 s  3 s
5 s  10 s  60 s

B  EGFP-RUNX2 Y413A

Pre-bleach  1 s  3 s
5 s  10 s  60 s
Figure 8. EGFP-RUNX2 NMTS Mutants in a Diffuse Subnuclear Distribution Are More Mobile than in the Other Subnuclear Distribution. SaOS-2 cells were transiently transfected with (A) EGFP-RUNX2 R383A, (B) EGFP-RUNX2 Y413A or (C) EGFP-RUNX2Δ361 and images for cells with a diffuse subnuclear distribution are shown. Cells were replated in coverslip chambers for FRAP analysis. Pre-bleached images are shown. The circles represent the entire photobleached areas in which the recovery rates were calculated. Shown images were captured before photobleaching and 1, 3, 5, 10 and 60 seconds after photobleaching. The white bars correspond to a scale of 10 microns.
A  EGFP-RUNX2 R383A

<table>
<thead>
<tr>
<th>Pre-bleach</th>
<th>1 s</th>
<th>3 s</th>
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<tbody>
<tr>
<td>5 s</td>
<td>10 s</td>
<td>60 s</td>
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B  EGFP-RUNX2 Y413A

<table>
<thead>
<tr>
<th>Pre-bleach</th>
<th>1 s</th>
<th>3 s</th>
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<tbody>
<tr>
<td>5 s</td>
<td>10 s</td>
<td>60 s</td>
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estimated mean half-time of recovery of 7.9 ± 0.5 seconds and a mean percent immobile fraction of 35.3 ± 5.2% calculated from two experiments (n=23) (Table 3).

These values are similar to those in Chapter 1. Moderate variations in quantitation are to be expected because of cellular or experimental differences. The half-time of recovery and percent immobile fraction of the R383A and Y413A NMTS mutants for the punctate distribution were not significantly different than those of wild-type RUNX2 (p > 0.05 using multiple pair-wise comparisons in the Tukey’s studentized range test and Bonferroni t-test). These results suggest that the NMTS mutations have no measurable effect on the mobility of RUNX2 (Table 3).

Interestingly, the half-time of recovery of the NMTS mutant proteins in a punctate distribution are significantly different than that of the diffuse distribution (p < 0.05). Furthermore, the percent immobile fractions for each of the three subnuclear distributions are significantly different (Table 3; p < 0.05). In addition, the mobility of the NMTS mutants in the diffuse subnuclear distribution is significantly less mobile than EGFP-RUNX2Δ361 (Table 3; compare percent immobile fractions; p < 0.05). This result suggests that the NMTS point mutations affect the mobility to a lesser extent than deleting the whole C-terminus. Therefore, a subset of the NMTS mutant proteins may be interacting with components of nuclear architecture and becoming immobilized for a short period of time. Furthermore, further decreases in mobility may be contributed by interactions of other regions of the C-terminus with nuclear components. The results show that RUNX proteins are more mobile in the diffuse distribution, where there are no foci formed and more immobile in the punctate distribution, where there are more foci (Table 3). One
Table 3. Average Recovery Rates for Wild-type EGFP-RUNX2 and the NMTS Mutants during FRAP Analysis in Living SaOS-2 Cells

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Subnuclear Distribution</th>
<th>n=</th>
<th>Half-time of recovery</th>
<th>Immobile fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-RUNX2</td>
<td>punctate</td>
<td>23</td>
<td>7.9 ± 0.5 s</td>
<td>35.3 ± 5.2%</td>
</tr>
<tr>
<td>EGFP-RUNX2Δ361</td>
<td>diffuse</td>
<td>10</td>
<td>&lt; 600 ms</td>
<td>0 %</td>
</tr>
<tr>
<td>EGFP-RUNX2 R383A</td>
<td>punctate</td>
<td>11</td>
<td>7.2 ± 0.8 s</td>
<td>42.2 ± 7.3%</td>
</tr>
<tr>
<td></td>
<td>punc/diff</td>
<td>7</td>
<td>-</td>
<td>21.2 ± 7.1%</td>
</tr>
<tr>
<td></td>
<td>diffuse</td>
<td>10</td>
<td>&lt; 900 ms</td>
<td>8.6 ± 2.8%</td>
</tr>
<tr>
<td>EGFP-RUNX2 Y413A</td>
<td>punctate</td>
<td>16</td>
<td>7.6 ± 0.5 s</td>
<td>50.2 ± 7.8%</td>
</tr>
<tr>
<td></td>
<td>punc/diff</td>
<td>11</td>
<td>-</td>
<td>27.8 ± 5.1%</td>
</tr>
<tr>
<td></td>
<td>diffuse</td>
<td>12</td>
<td>&lt; 800 ms</td>
<td>5.3 ± 4.0%</td>
</tr>
</tbody>
</table>
interpretation of these results may be that at the subnuclear foci the C-terminus of RUNX transcription factors binds to a component of the nuclear architecture and retains RUNX2 to regulate gene transcription. However, the entire NMTS and/or other regions of the C-terminus (e.g., co-regulatory protein interaction domains) may be necessary to immobilize RUNX proteins.

Because differences in the subnuclear distribution of the RUNX2 proteins were observed, we assessed whether these differences may be due to variation in fluorescence intensities of the nuclei. Therefore, the mean pre-bleached fluorescence intensities of the entire nuclei were calculated for EGFP-RUNX2 and EGFP-RUNX2Δ361 (Figure 9A), and for each of the subnuclear distributions of the EGFP-RUNX2 R383A (Figure 9B) and EGFP-RUNX2 Y413A (Figure 9C) mutants. The integrated fluorescence intensity of each nucleus was determined by multiplying the intensity of the nucleus by the number of pixels in which the intensity was integrated. The exposure time of each image was 200 ms. The results show that the diffuse distribution correlates with a lower level of fluorescence intensity. Moreover, the punctate distribution correlates with a higher level of fluorescence (Figure 9). Thus, these results suggest that the concentration of RUNX proteins in the nucleus may have an effect the formation or size of the punctate foci.
Figure 9. Punctate Subnuclear Distribution Correlates with a Higher Fluorescence Intensity. FRAP analysis of the mean pre-bleached fluorescence intensity of the nuclei was graphed for (A) EGFP-RUNX2 and EGFP-RUNX2Δ361, and each subnuclear distribution of (B) EGFP-RUNX2 R383A and (C) EGFP-RUNX2 Y413A. In (A), EGFP-RUNX2 expressing cells were only punctate (n=23) and EGFP-RUNX2Δ361 expressing cells were only diffuse (n=10). In (B), the number of nuclei observed (n) expressing EGFP-RUNX2 R383A in the punctate distribution equals 11, in the punctate/diffuse distribution is 7, and in the diffuse distribution is 10. In (C), the number of nuclei observed (n) expressing EGFP-RUNX2 Y413A in the punctate distribution is 16, in the punctate/diffuse distribution is 11, and in the diffuse distribution is 12. Bar graphs equal mean ± SEM.
**A**

![Integrated pre-bleached intensity of entire nucleus](chart)

- **EGFP-RUNX2**
  - punctate
- **EGFP-RUNX2Δ361**
  - diffuse

**B**

![Integrated pre-bleached intensity of entire nucleus](chart)

- **EGFP-RUNX2 R383A**
  - punctate
  - punctate/diffuse
  - diffuse

**C**

![Integrated pre-bleached intensity of entire nucleus](chart)

- **EGFP-RUNX2 Y413A**
  - punctate
  - punctate/diffuse
  - diffuse
DISCUSSION

In this study, we show that two specific point mutations in the NMTS of RUNX2 (i.e., R383A and Y413A) decrease association with the nuclear matrix. However, these mutations have no measurable effect on the mobility and subnuclear distribution of RUNX2. The similarities between the mobility of wild-type RUNX2 and the RUNX2 NMTS point mutants suggest that these single point mutations may be less effective in decreasing the nuclear matrix association than is necessary to observe a difference in the mobility in living cells. Several possible interpretations could be drawn from these results. The entire NMTS may be involved in association with subnuclear domains. It is plausible that creation of either different single point mutations in the NMTS, a combination of several NMTS point mutations or an NMTS internal deletion may result in a difference in the mobility compared to wild-type RUNX2 protein. FRAP analysis could be performed on these alternative mutants to determine if this interpretation is valid. An alternative interpretation, which is supported by the result that there is no difference in the mobility with the NMTS mutants, could be that the interaction of the C-terminus of RUNX2 with co-regulatory proteins may be more important than its association with the nuclear matrix to account for the decrease in mobility of RUNX2 compared to that of the C-terminal truncated RUNX2 (EGFP-RUNX2Δ361) observed in Chapter 1. This conclusion is consistent with the findings that several co-regulatory factors interact with RUNX proteins in their C-termini and that the interaction sites were deleted in EGFP-RUNX2Δ361 (see General Introduction, Figure 3). However, whether the
NMTS mutations altered interaction with co-regulatory proteins has not been evaluated except with Smad and YAP proteins (Zaidi et al., 2002; unpublished results from S. K. Zaidi). A region of the Smad interaction site overlaps with the NMTS (see General Introduction, Figure 3). However, the Y428A NMTS mutation in the RUNX2 native protein did not affect the interaction of RUNX2 with Smad proteins by co-immunoprecipitation assays (Zaidi et al., 2002). Interaction of the NMTS mutant proteins with other co-regulatory proteins could be established by co-immunoprecipitation assays to validate this interpretation. Another possibility is that the large immobile fraction of the punctate subnuclear distribution could represent association of RUNX proteins with stable subnuclear foci by interaction with transcription factor complexes. This concept is supported by the result that RUNX proteins are more mobile in the diffuse subnuclear distribution, which does not contain subnuclear foci.

It would be expected that the mutations in the NMTS would result in an increase in the mobility of RUNX2 proteins. However, we observed no difference in the mobility between the NMTS mutants and wild-type RUNX2. Two considerations may help to explain these results. First, the point mutations did not completely abolish nuclear matrix association. Therefore, deletion of the entire NMTS may have a more substantial effect on the mobility of RUNX proteins. Second, RUNX proteins may transiently associate with subnuclear foci and therefore be in constant flux with subnuclear foci. In addition, the C-terminus of RUNX proteins may associate with co-regulatory proteins that form a large multi-subunit complex, which may slow down the mobility of RUNX proteins.
Two mechanisms for the movement of nuclear proteins are plausible, a
diffusional mechanism, which does not require energy or a non-diffusion, directional
mechanism, in which proteins may be targeted by a specific signal (Misteli, 2001;
Kruhlak et al., 2000; Phair and Misteli, 2000; Shopland and Lawrence, 2000). While
our results show that RUNX proteins are targeted to certain subnuclear sites, they
may move to these sites initially by either a directed movement or a more random
diffusion followed by specific retention. It would be useful to distinguish between
these two mechanisms in the future, and to examine the role of the NMTS specifically
in the possibility of directed movement.

The differences observed in each of the subnuclear distributions for the
mobility of RUNX2 may reflect an increase in protein concentration. This is
supported by the results that the lower fluorescence intensity correlates with a diffuse
subcellular distribution and that the higher fluorescence intensity, which represents an
increase in protein concentration, correlates with a punctate distribution. These
results suggest that the formation of punctate subnuclear foci may be related to the
level of protein in the nucleus. It should be noted that the punctate RUNX foci are
observed with endogenous levels of expression in fixed SaOS-2 cells (see Chapter 1,
Figure 3) and fixed human osteoblastic cells (Prince et al., 2001), which has a similar
appearance as EGFP-RUNX1 and EGFP-RUNX2 foci. An increase in protein
concentration may result in an increase in flow of freely mobile RUNX proteins into
the subnuclear foci, thereby increasing the size and intensity of the subnuclear foci.

The majority of the RUNX subnuclear foci are associated with sites of
transcription (see Chapter 1, Figure 6), indicating that these punctate foci may
represent multisubunit complexes. The increase in the number of punctate foci as RUNX protein concentration is increased may reflect an increase in number of transcription factor complexes, and therefore an increase in gene expression. However, not all of the RUNX foci are associated with sites of transcription. RUNX sites not actively involved with transcription may be sites of storage or transcriptional repression.

Several limitations of the FRAP experiments exist. First, the speed of the CCD camera was slower than the mobility of the proteins in a diffuse distribution. Therefore, two formulas were necessary to calculate the half-time of recovery. Second, a light fluorescence microscope was used instead of a confocal microscope for the FRAP analysis, and thus the out-of-focus light was not removed from the images. Finally, the mobility of wild-type RUNX2 in the punctate/diffuse and diffuse subnuclear distributions were not determined to compare to the punctate distribution. Therefore, further studies may be necessary to conclusively determine whether the NMTS is responsible for the retention of RUNX proteins at subnuclear foci, since the question still remains whether nuclear matrix association is the cause of immobilization of RUNX factors.
CHAPTER 3

The RUNX Inducible Osteocalcin Promoter Expresses Enhanced Green Fluorescent Protein Specifically in the Osteoblastic Lineage of Transgenic Mice
The OC-EGFP transgenic mice were generated and characterized in collaboration with Thomas Owen, Ph.D. at Pfizer, Groton, CT.
INTRODUCTION

RUNX2 transcription factors are highly expressed in bone cells and regulate several bone specific genes, including the osteocalcin (OC) gene, during osteoblast (OB) differentiation. To assess the in vivo regulation of osteocalcin by RUNX proteins, we generated transgenic mice expressing enhanced green fluorescent protein (EGFP) under control of the OC promoter. The OC-EGFP mice create a useful tool for evaluating the in vivo regulation of osteocalcin by RUNX2 and for developing potential new drugs for the treatment of bone diseases, such as osteoporosis.

The osteocalcin protein is a ~10-kDa protein, which is synthesized only in osteoblasts (OBs) and is secreted from OBs into the mineralized bone extracellular matrix. OC is undetectable in early osteoprogenitor cells in bone and its expression is induced in committed osteoblasts. Preosteocytes and osteocytes in mature bone continue to express OC at significant levels (Lian et al., 2001). In early stages of normal osteoblast differentiation in vitro (day 3), osteoblasts are in a proliferation period, where they express high levels of histone and collagen type I genes, but do not express OC. Basal levels of OC are expressed in post-proliferative osteoblasts. OC mRNA and protein expression are induced in cultured OBs in late stages of osteoblast differentiation during bone matrix maturation (day 12 of cultures) and mineralization (day 19 of cultures). During these stages, mineralized bone nodules form in OB cultures and a temporal expression of other bone-specific phenotypic markers, alkaline phosphatase and osteopontin, are also induced (Owen et al., 1991).

Basal expression of the OC gene involves several responsive elements in the proximal promoter, including the TATA box (located from -42 to -39) and the OC
Figure 1. Schematic of the Rat Osteocalcin Gene Promoter. Regulatory sequences and their cognate binding factors are shown for the active osteocalcin gene transcribed in differentiated osteoblasts. The TATA box is the site of interaction with the transcription machinery. OC Box I is a 24 nt domain containing a homeodomain binding site (e.g., Msx-2, Dxl-5) and a site for an osteoblast-specific binding protein (OCBP1). Other functional regulatory elements include glucocorticoid response element (GRE) and one responsive to transforming growth factor (TGRE). Recognition motifs for RUNX proteins are shown with sites A and B flanking the vitamin D response element (VDRE) and site C located in the proximal promoter. During basal transcription, the VDRE is occupied by AP-1 and YY1 proteins as indicated. In the active gene, the positioned nucleosome is shown and the DNase I hypersensitive sites (DHS) spanning the proximal and distal regulatory domains are illustrated.
Osteocalcin gene promoter
box I (located from -99 to -76) (Figure 1). The TATA box binds TFIIB, TFIID and the transcription machinery. The homeodomain proteins (e.g., Msx-2, Dlx-5 and CDP/cut) repress OC expression by interaction with the OC box in early stages of OB differentiation (Hoffmann et al., 1994; Towler et al., 1994b; Ryoo et al., 1997; van Gurp et al., 1999). However, heterodimerization of Msx-2 with the homeodomain protein Dlx-5, which is expressed in differentiated OBs has been shown to disrupt DNA binding and thereby relieve the repression by homeodomain proteins (Lian et al., 2001; Ryoo et al., 1997; Zhang et al., 1997; Newberry et al., 1998). The osteoblast specific binding complex (OCBP1) also interacts with the OC box to induce basal transcription (Stein et al., 1997; Hoffmann et al., 1996).

Transcriptional activation of the OC promoter critically involves the association of RUNX proteins with the three RUNX binding sites (Sites A, B, and C) as well as the enhancer vitamin D response element (VDRE, -461 to -445), which binds the vitamin D receptor (VDR)-retinoid X receptor (RXR) heterodimer complex (Figure 1; Stein et al., 1997). During basal expression or when the OC promoter is inactive, the VDRE is occupied by the YY1 protein and c-jun and c-fos AP-1 factors, which inhibit OC expression (Lian et al., 2001). Induction of OBs with 1,25-(OH)₂ vitamin D₃ results in the upregulation of OC. Maximal expression of the OC promoter results from interaction of the VDR/RXR heterodimer with the VDRE upon vitamin D₃ induction. The VDRE is located in between RUNX sites A and B (-604 to -599 and -440 to -435) in the distal promoter, which are required for induction of the OC promoter by vitamin D₃ (Javed et al., 1999; Stein et al., 1997). RUNX Site C (-135 to -130) is located in the proximal promoter. The RUNX sites were originally
identified as nuclear matrix protein (NMP-2) associated sites (Bidwell et al., 1994).
Initial characterization of NMP-2 revealed that it is a RUNX-related transcription factor further identified as RUNX2/AML3, which is highly expressed in osteoblasts (Merriman et al., 1995; Banerjee et al., 1997). RUNX proteins associate with the nuclear matrix, linking the OC promoter DNA to the nuclear architecture (Zeng et al., 1997). This complexity of transcriptional activity of OC during OB differentiation in vitro necessitates an understanding of OC expression in vivo.

Transgenic and knockout mice are important for determining the in vivo function of OC in bone differentiation. However, the function of osteocalcin is still unknown. OC-deficient mice are normal at birth, viable, fertile and have no skeletal patterning defects or ectopic bone formation (Ducy et al., 1996). These mutant mice develop a higher bone density compared to their wild-type littermates over time, indicating that the lack of osteocalcin leads to increased bone formation. These results may provide a therapeutic model for osteoporosis, which is characterized by a decrease in bone mass due to increased bone resorption and decreased bone formation.

Expression of the OC promoter fused to a reporter gene in transgenic mice can create a useful tool for OC expression as a marker to assess osteoblast growth and differentiation and bone metabolism in vivo and to examine the regulation of OC by RUNX proteins in vivo. Therefore, we used enhanced green fluorescent protein (EGFP) as a reporter gene driven by the OC promoter to produce OC-EGFP transgenic mice, since EGFP has intrinsic fluorescence properties, which can be visualized by fluorescence microscopy of whole bone or ex vivo osteoblast cultured
cells. Our results show that EGFP is expressed from the OC promoter in a cultured osteosarcoma cell line, but not in a kidney cell line, and is induced by vitamin D3. Furthermore, EGFP is expressed in tails of OC-EGFP transgenic mice, which allows for phenotyping by visualization of the green fluorescent tail using fluorescence microscopy. Our results demonstrate that OC-EGFP transgenic mice specifically express EGFP in osteoblasts and osteocytes in bone tissues. Moreover, EGFP is expressed in mineralized bone nodules of differentiated bone marrow derived from transgenic mice. Taken together, these results show that active osteoblasts are marked with EGFP, which can be easily visualized in tissue sections and differentiated osteoblasts, suggesting that the OC-EGFP transgenic mice are a good model for studying the in vivo effects of RUNX on OC expression and for developing potential drugs therapies for bone diseases that effect OB growth, differentiation and metabolism in vivo.
MATERIALS AND METHODS

Cell Culture

Rat osteosarcoma ROS 17/2.8 cells (Henry Korenberg, Massachusetts General Hospital, Boston, MA) and monkey kidney COS-7 cells (American Type Culture Collection (ATCC), Manassas, VA) were maintained in completed F12 with 5% (v/v) fetal bovine serum (FBS) or DMEM medium with 10% FBS, respectively, at 37 °C in a humidified 5% CO₂ incubator. Both media contained 100 Units/ml Penicillin, 100 μg/ml Streptomycin and 2 mM L-glutamine and was changed every 2-3 day.

Plasmids

pSROEGFP containing the -1.7 kb rat osteocalcin (OC) promoter fused to EGFP reporter gene was cloned in a 3-step process (Figure 2). The first step was to insert a palindromic adaptor containing a HindIII site into the NheI site of pEGFP-C3. To insert the adaptor, the pEGFP-C3 vector (Clontech, Palo Alto, CA) was digested with NheI and the linearized vector was gel extracted using QIAGen gel extraction kit (Qiagen, Valencia, CA). The adaptor was produced from a 12-nt oligo with the palindromic sequence 5’-CTAGCAAGCTTG-3’ which contained two NheI overhangs on either side of a HindIII site. The oligo was heated to 65 °C for 5 minutes, slow cooled to room temperature and placed on ice to self-hybridize the oligo. The adaptor was ligated to the NheI digested pEGFP-C3 vector resulting in the clone pEGFP-C3-HindIII. The second step was to remove the HindIII site from the multiple cloning sequence by self-ligating the BgIII/BamHI digested pEGFP-C3-
Figure 2. Generation of the Osteocalcin Promoter-EGFP Expression Vector. A three-step cloning process was used to produce the pSROEGFP construct, which contained the enhanced green fluorescent protein (EGFP) reporter gene fused to the 1.7 kb rat osteocalcin (OC) promoter. First, a palindromic adaptor (sequence shown below) containing an internal HindIII site flanked by two NheI overhangs was inserted into the pEGFP-C3 vector (Clontech; top left), which was digested with NheI. The resulting clone was named pEGFP-C3-HindIII (top right) and was subsequently digested with BglII and BamHI and self-ligated to remove the HindIII site in the multiple cloning sequence. The resulting vector was called pEGFP-HindIII (middle right). pEGFP-HindIII and pSROCAT (middle left), which contains the chloramphenicol acetyl transferase (CAT) reporter gene driven by the OC promoter, were digested with HindIII and MluI. The HindIII/MluI fragment of pSROCAT containing the OC promoter (pSRO) was fused to the HindIII/MluI fragment of the EGFP gene replacing the CAT gene with the EGFP gene and creating the final construct pSROEGFP (bottom). Restriction sites used for digestion are in bold print.
Digest with Nhel
Ligate adaptor

Palindromic Adaptor Sequence
5'-CTAGCAAGCTTG-3'
3'-GTTCCAACGATC-5'

Nhel  HindIII  Nhel
HindIII vector. The resulting clone was pEGFP-HindIII. The last step was to clone the EGFP cDNA into pSROCAT, which contains the −1.7 kb rat osteocalcin (OC) promoter fused to the chloramphenicol acetyl transferase (CAT) gene (Hoffman et al., 1996), by replacing the CAT gene with the EGFP gene. pSROCAT and pEGFP-HindIII were digested with HindIII and MluI. The HindIII/MluI EGFP fragment and the OC promoter and vector backbone (pSRO) were gel extracted using the QIAquick gel extraction kit (Qiagen) and ligated together to generate pSROEGFP. pSROEGFP was sequenced using Sequenase version 2.0 DNA sequencing kit (Amersham Pharmacia, Piscataway, NJ) with the SITE C top strand primer (5'-GTCACCAACCACACGATCCCTTG-3').

In situ Immunofluorescence

ROS 17/2.8 and COS-7 cells were grown on 0.5% (w/v) gelatin-coated coverslips and were transiently transfected using DEAE-Dextran method. Briefly, 0.025 mg/ml of DEAE-Dextran and 0.05 mg/ml chloroquine were mixed with 10 ml of serum-free F12 or DMEM media. 1 μg of pSROEGFP DNA was added to 0.5 ml of media plus DEAE-Dextran and Chloroquine and incubated with the cells for 3 hours at 37 °C rocking plates every 30 minutes. The media was aspirated and the cells were shocked for 90 seconds with 10% glycerol in F12 or DMEM media. Cells were washed twice with 1X PBS and feed with F12 medium supplemented with 5% charcoal-stripped serum for ROS cells or DMEM supplemented with 10% charcoal-stripped serum for COS-7 cells either with or without 10⁻⁸ M 1,25-(OH)₂-vitamin D3 treatment and incubated at 37 °C for 24 hours. Cells were fixed using 3.7%
formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS and DNA was stained with 0.05 µg/ml DAPI. Cells were mounted in Vectashield antifade mounting media (Vector Laboratories, Burlingame, CA). Fluorescence images were captured using a Zeiss ICM 405 microscope with a 40X Nikon Plan-Apo 40 oil objective (1.0 N.A.) or a 100X Zeiss F100 oil objective (1.25 N.A.), a 100 W Hg lamp and a Zeiss MC80 Dx 35 mm camera using Kodak Ektachrome 400X film (Eastman Kodak Company, Rochester, NY). The software applications to prepare scanned images were Adobe Photoshop and Microsoft PowerPoint.

Northern Blot Analysis

ROS 17/2.8 cells were plated at 0.6 x 10^6 cells/100 mm plate and incubated until 80% confluent. Cells were either untransfected or transiently transfected with 10 µg pSROEGFP and 40 µl of Superfect reagent (Qiagen) and allowed to recover for 4 hours following transfection in completed F12 media. The medium was replaced with F12 supplemented with 2% charcoal-stripped serum and antibiotics either with or without 10^{-8} M 1,25-(OH)_2-vitamin D3 treatment and incubated for 24 hours at 37 °C. Cells were washed and scraped in 1 ml PBS. mRNA was isolated from the cell pellets using the PolyA mRNA Isolation kit (Roche, Indianapolis, IN). Briefly, the cell pellets were resuspended in 1.5 ml lysis buffer mix and DNA was sheared mechanically by passing through a 21-gauge needle on ice. 1.5 µl of biotin-labeled oligo (dT)20 was added to the cell lysate. The cell lysate was then added to the washed streptavidin-coated magnetic particles and incubated at 37°C for 5 minutes. The magnetic particles were separated from the fluid using a magnet and washed 3
times. The mRNA was eluted from the magnetic particles with 25 µl of redistilled water. 0.7 µg mRNA was lyophilized and resuspended in 14 µl of sample buffer (1.6 µl 10X MOPS, 2.8 µl 37% formaldehyde, 8 µl 100% formamide, 2.4 µl bromophenol blue dye, 0.2 µl 10 mg/ml ethidium bromide). 0.7 µg of mRNA was loaded on a 1% agarose gel and 6.29% formaldehyde in 1X MOPS (0.2 M MOPS, 0.05 M sodium acetate, 0.01 M Na₂EDTA). mRNA separated on the gel was transferred onto a Hybond-N+ nylon membrane (Amersham Pharmacia, Piscataway, NJ) overnight in 20X SSC. The membrane was crosslinked for 30 seconds with UV light and prehybridized with 10 ml prehybridization solution (for 50 ml total volume, 1.25 ml of 1 M KPO₄ (pH 7.4), 12.5 ml of 20X SSC, 10 µl of 10 mg/ml salmon sperm DNA (Sigma, St. Louis, MO), 25 ml of 100% formaldehyde and 5 ml of 10% SDS). 84 ng of EGFP probe (NheI/XhoI fragment of pEGFP-C3) was boiled with 10 µl 9-mer random primer from the Prime it II kit (Stratagene, La Jolla, CA) in a 33 µl total volume for 5 minutes and slow cooled to room temperature. 10 µl 5X dCTP primer buffer (Stratagene, La Jolla, CA), 50 µCi ³²P-dCTP (Perkin Elmer, Boston, MA) and 5 Units of Klenow enzyme (1 Unit/µl) (Stratagene, La Jolla, CA) were added to the probe and incubated 15 minutes at 37 °C. Excess ³²P was removed by passing the probe through a Sephadex G-25 Quick Spin column (Roche, Indianapolis, IN). The probe was resuspended in 50 µl hybridization buffer (prehybridization buffer and 10% Denhardt solution), boiled for 5 minutes, added to 10 ml of hybridization solution and incubated at 42 °C with the membrane overnight in a hybridization oven. The blot was washed 3 times with a low stringency buffer (2X SSC, 0.1% SDS) at room temperature and exposed to film 2 hours to overnight at -70 °C. Blots were
stripped with 0.1X SSC and 0.5% SDS by boiling for 5 minutes and reprobed with the OC probe {BamHI/EcoRI fragment of the rat OC plasmid pOC3.4 (Lian et al., 1989)} and the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe {HindIII/EcoRI fragment of the pmdm23 plasmid (Nakao et al., 1994)}.

**FACS Analysis**

ROS 17/2.8 cells were plated at 0.6 x 10^6 cells/100 mm plate and incubated until 80% confluent. Cells were transiently transfected with 10 µg pSROEGFP and 40 µl of Superfect reagent (Qiagen) and allowed to recover for 4 hours following transfection in completed F12 media. The medium was replaced with F12 supplemented with 2% charcoal-stripped serum and antibiotics either with or without 10^{-8} M 1,25-(OH)2-vitamin D3 treatment and incubated for 24 hours at 37 °C. Cells were trypsinized using 1 ml Trysin-0.25% (Invitrogen, Carlsbad, CA) for 1-2 minutes at 37 °C and counted. 1 x 10^6 cells were centrifuged at 1000 rpm for 5 minutes and resuspended in 400 µl PBS. Flow cytometric analysis was performed by the Core Flow Cytometry Facility at the University of Massachusetts Medical School, Worcester, MA. Twenty thousand cells were counted to determine the percent of EGFP expressing cells with high, medium, or low expression levels. Calculations of percent EGFP expressing cells were averages from two experiments ± S.D. (n=5).
Preparing the Rat Osteocalcin Promoter-Enhanced Green Fluorescent Protein (OC-EGFP) DNA Fragment for Microinjection to Generate OC-EGFP Transgenic Mice

pSROEGFP was digested with BamHI and MluI to release the OC-EGFP DNA fragment and ApaLI to cut the vector backbone into smaller fragments since the OC-EGFP fragment and the backbone were of similar sizes. The OC-EGFP fragment needed to be extremely pure for microinjection in the pronuclear stage embryos, therefore the digested DNA was then extracted by phenol: chloroform (1:1) and the residual phenol:chloroform was extracted by ethyl ether (equilibrated with water) (1:1 vol). The residual ethyl ether was evaporated by incubation in a 70 °C water bath for 4-5 minutes. The DNA was ethanol precipitated three times and loaded on a 0.7% agarose gel. The OC-EGFP fragment was gel extracted with QIAquick gel extraction kit (Qiagen). The OC-EGFP DNA was then further purified using Elutip columns (Schleicher and Schueil, Keene, NH) and the transgenic mice were generated as described in (Owen et al., 2001). Briefly, the purified OC-EGFP DNA was microinjected into pronuclear stage FVB/N embryos at a concentration of ~3.0 ng/μl in 10 mM Tris-Cl pH 7.4 and 0.1 mM EDTA. Microinjected embryos were transferred to pseudopregnant CD-1 females. At weaning, genomic DNA was isolated from by phenol: chloroform extraction and the founder transgenics were identified by PCR using primers that span the 3' end of the OC promoter and the 5' end of the EGFP gene. Founder mice were bred to FVB/N mates and the transgenic lines were maintained. Transgenic mouse line “F” was the only line in which the EGFP fluorescence could be visualized by fluorescence microscopy for phenotyping.
Histology

Histology was performed as described in Owen et al. (2001). Briefly, mice were sacrificed by CO₂ inhalation. Tissues were removed, fixed in 4% paraformaldehyde and stored in 0.5% paraformaldehyde at 4 °C until processing. For cryosectioning, tissues were mounted in OCT (Miles Inc., Elkhart, IN; 10.24% (w/w) polyvinyl alcohol; 4.26% polyethylene glycol; 85.50% nonreactive ingredients) and frozen using a Gentle Jane snap freezing system (Instrumedic Inc., Hackensack, NJ). Frozen blocks were mounted on a Leica CM3000 cryostat and 5 μm sections were cut. Sections were imaged using an Olympus BH-2 fluorescence microscope system (Melville, NY).

Bone Marrow Cell Culture

Bone marrow cultures were generated as described in Owen et al. (2001). Briefly, mouse femur and tibia bone marrow from 8 week old transgenic mice was extracted, triturated, passed through a 100 μm mesh filter and plated in complete MEMα media supplemented with 15% fetal calf serum (FCS) and antibiotics in 6-well dishes at a density of 3 x 10⁶ cells/well. Cells were fed on day 4 by replacing one-half of the plating media and from day 8 onward were fed with MEMα supplemented with 10% FCS, 50 μg/ml L-ascorbate and 2 mM sodium inorganic phosphate to induce osteoblast differentiation.
RESULTS

Generation of Osteocalcin Promoter-EGFP Reporter Construct

Transgenic mice expressing EGFP under control of the osteocalcin (OC) promoter may be useful to study the in vivo function of RUNX proteins in bone development and to develop drugs to treat bone diseases such as osteoporosis and osteopenia. Therefore, to produce transgenic mice expressing EGFP under the control of the −1.7 kb rat osteocalcin (OC) promoter, we generated an OC-EGFP expression construct termed pSROEGFP (Figure 2 and 3A). pSROEGFP was generated by 3-step cloning process, which replaced the chloramphenicol acetyl transferase (CAT) gene in pSROCAT (Hoffman et al., 1996) with enhanced green fluorescent protein (EGFP) (Figure 2). First, an adaptor containing a HindIII site was inserted 5’ of the EGFP start site in the pEGFP-C3 vector (Clontech) so that the EGFP gene could subsequently be cloned into the HindIII site at the 3’end of the OC promoter. Second, the HindIII site was removed from the multiple cloning sequence of the resulting pEGFP-C3-HindIII construct, so only a single HindIII site was remaining. Lastly, the resulting pEGFP-HindIII construct and pSROCAT were digested with HindIII and MluI and the EGFP gene was fused to the 3’ end of the OC promoter replacing the CAT gene with the EGFP gene (Figure 2).
Expression of EGFP Under the Control of the Osteocalcin Promoter Is Induced by Vitamin D3

To assess whether the pSROEGFP construct tissue-specifically expressed EGFP under control of the OC promoter, we transiently transfected rat osteosarcoma ROS 17/2.8 cells and monkey kidney COS-7 cells with pSROEGFP (Figure 3A) and treated the cells with 1,25-(OH)₂ vitamin D₃ for 24 hours. Since OC is expressed only in osteoblasts, we expect that EGFP would be specifically expressed in ROS 17/2.8 cells, but not COS-7 cells. The results show that EGFP is expressed from the OC promoter in ROS 17/2.8 cells, but not in COS-7 cells by in situ fluorescence microscopy. Furthermore, as a positive control, EGFP was express by a CMV promoter in COS-7 cells. Taken together, these results suggest that EGFP under control of the OC promoter is specifically expressed in bone cells in vivo (Figure 3B).

An overall induction of the OC promoter by vitamin D₃ was not observed by in situ immunofluorescence since the number of transfected cells by DEAE-Dextran transfection method was very low (transfection efficiency= 1%) and the expression levels of EGFP varied from cell to cell either with or without vitamin D₃. However, since the endogenous OC promoter is induced over basal levels by vitamin D₃ (Lian et al., 2001), we expected that the pSROEGFP expression construct would be induced by vitamin D₃. Therefore, to determine whether expression of EGFP mRNA under control of the OC promoter was indeed induced by vitamin D₃, we performed northern blot analysis of ROS 17/2.8 cells transfected with pSROEGFP in the presence or absence of vitamin D₃. The results show a 17-fold induction of EGFP.
Figure 3. EGFP Is Expressed Under Control of the Rat Osteocalcin Promoter in Osseous, but Not in Non-Osseous Cell Lines. (A) Schematic of the pSROEGFP expression construct containing the −1.7 kb rat osteocalcin promoter fused to enhanced green fluorescent protein (EGFP) is illustrated. Shown are binding sites for RUNX (Sites A, B and C), the vitamin D responsive element (VDRE), CCAAT/ enhancer binding protein (C/EBP), and the OC box I and the TATA box. (B) pSROEGFP was transiently transfected in rat osteosarcoma ROS 17/2.8 cells and monkey kidney COS 7 cells. EGFP (green fluorescence, left panels) is expressed in ROS 17/2.8 cells, but not in COS-7 cells. DAPI (0.05 μg/ml) stains the DNA in the nucleus (right panels). Images shown are from 24-hour 1,25-(OH)₂ vitamin D₃ treatment. No difference was observed between the fluorescence of the untreated and the 24-hour vitamin D₃ treatment for both cell types.
-1.7 kb Rat Osteocalcin Promoter-EGFP
Figure 4. EGFP mRNA Expressed from the Osteocalcin Promoter Is Induced by Vitamin D3 in Rat Osteosarcoma Cells. ROS 17/2.8 cells were either untransfected or transiently transfected with pSROEGFP and treated either with or without vitamin D3 in charcoal-stripped serum for 24 hours. Cells were harvested and Poly A mRNA was isolated from cell pellets. 0.7 μg of mRNA was loaded on a 1% agarose-6.29% formaldehyde gel and was transferred onto a nylon membrane. The membrane was probed with 32P-labeled EGFP, OC and GAPDH DNA probes and the autoradiographs are shown. The bands were quantitated using the phosphoimager. Fold vitamin D3 induction was determined by normalizing to GAPDH expression levels.
Vitamin D3 Induction

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mRNA expression by vitamin D3 in cells expressing pSROEGFP using a probe for EGFP (Figure 4). As a control, the expression of endogenous OC mRNA was induced 10 to 73-fold by vitamin D3 (Figure 4), which is consistent with previous results (Breen et al., 1994; Lian et al., 2001). The fold induction was normalized to GAPDH mRNA levels. Furthermore, to determine whether the EGFP protein levels were induced by vitamin D3, ROS 17/2.8 cells were either untransfected or transfected with pSROEGFP and treated either with or without vitamin D3. EGFP expressing cells were sorted by FACS analysis for high, medium and low expression levels (Figure 5). The FACS analysis dot plots show an increase in the number of cells with high expression levels after vitamin D3 treatment (Figure 5A). Calculations of the percent of EGFP expressing cells show no change in the number of low expressing cells (6-7% of population; Figure 5B). However, an increase in the percent of medium expressing cells (from 4% to 6% of the population) and high expressing cells (from 1% to 4% of the population) was observed in the presence of vitamin D3 (Figure 5B). This increase by vitamin D3 treatment was only observed by dividing the population into low, medium and high expressing cells, which suggests a reason why no overall induction was observed with vitamin D3 treatment in the entire population of cells by immunofluorescence microscopy. Taken together, these results suggest EGFP mRNA and protein levels are induced by vitamin D3. Furthermore, the OC-EGFP construct expresses in specifically in bone cells in vitro. Therefore, the OC-EGFP construct is a good candidate to generate transgenic mice.
Figure 5. EGFP Protein Expression from the Osteocalcin Promoter Is Induced by Vitamin D3. ROS 17/2.8 cells were either untransfected or transiently transfected with pSROEGFP and treated either with or without vitamin D3 in charcoal-stripped serum for 24 hours. Cells were harvested for FACS analysis by trypsinization and resuspended in PBS without fixation. (A) Dot plots from the FACS analysis are shown for untransfected ROS 17/2.8 cells (top), pSROEGFP transfected cells without vitamin D3 treatment (left), or pSROEGFP transfected cells with vitamin D3 treatment (right). The EGFP expressing cells in the dot plots were sorted into low, medium (med) and high fluorescence levels (boxes). Several cells were observed with a very high expression level (high box on top) with vitamin D3 treatment and were included in the calculations for the high expressing cells. (B) From the dot plots above the mean percentage of EGFP expressing cells in each of the expression levels with and without vitamin D3 treatment was calculated from two experiments (n=5). The error bars represent standard deviations.
Preparation of the Osteocalcin Promoter-Enhanced Green Fluorescent Protein (OC-EGFP) DNA Fragment for Generation of OC-EGFP Transgenic Mice

Transgenic mice expressing EGFP under control of the OC promoter would be expected to specifically express EGFP proteins in bone cells, since OC is a bone-specific protein. EGFP provides a sensitive marker for bone formation, which can be visualized by fluorescence microscopy in vivo. Therefore, we prepared the BamHI/MluI OC-EGFP DNA fragment by digestion with BamHI, MluI and ApaLI (Figure 6). Digestion with ApaLI was utilized to cut the vector backbone into smaller fragments since the backbone had a similar molecular weight as the OC-EGFP fragment. The BamHI/MluI OC-EGFP fragment was purified by phenol: chloroform extraction, ethyl ether extraction, ethanol precipitations, gel extraction and column purification (Figure 6). The purified OC-EGFP fragment was microinjected into pronuclear stage embryos, which were then transferred into pseudopregnant female mice. Although 5-8 mouse lines were positive by PCR for the OC-EGFP transgene, only line “F” produced visually detectable levels of EGFP in the tails of transgenic mice for phenotyping by fluorescence microscopy (Figure 7; Owen et al., 2001). Expression of EGFP in cryosections of the tibia, calvarium, femur and kidney of 28-day old transgenic and wild-type mice was assessed by fluorescence microscopy (Figure 8; Owen et al., 2001). The results demonstrate that EGFP was specifically expressed in osteoblasts and osteocytes of bone tissues in transgenic mice. Furthermore, no EGFP expression was observed in the kidney of transgenic mice or in the femur or kidney of wild-type mice (Figure 8; Owen et al., 2001). Northern blot analysis was performed on OC-EGFP mRNA and endogenous OC mRNA extracted
Figure 6. Protocol for Preparing OC-EGFP DNA Fragment to be Microinjected into Female Pseudopregnant Mice. The pSROEGFP clone was digested with BamHI and MluI to release the OC-EGFP fragment of DNA from the vector backbone. However, the vector backbone fragment was about the same size as the OC-EGFP fragment. Therefore, the vector backbone was digested with ApaLI to cut it into smaller fragments. The DNA in the digestion reaction was extracted with phenol:chloroform (1:1). The residual phenol:chloroform was then extracted with ethyl ether, which was equilibrated with water. The residual ethyl ether was evaporated by incubation in a 70 °C water bath for 4-5 minutes with the lid open.

The DNA was then ethanol precipitated with 100% ethanol, centrifuged and washed with 70% ethanol three times. The DNA was dissolved in TE and loaded on a 0.7% agarose gel and the separated OC-EGFP DNA fragment was gel extracted. The OC-EGFP DNA was further purified using Elutip columns. The purified DNA was microinjected into pronuclear stage embryos, which were then transferred to pseudopregnant female CD-1 mice to produce the OC-EGFP transgenic mice.
Protocol for Preparing OC-EGFP DNA fragment to Produce Transgenic Mice

Clone pSROEGFP

Digest pSROEGFP with BamHI, MluI and ApaLI

Phenol:Chloroform (1:1) Extract DNA

Ethyl Ether (equilibrated with H2O) Extract DNA (1:1 vol)

Heat in 70°C water bath, 4-5 minutes, with lid open to boil excess ether

Ethanol Precipitate (3 times)

Load DNA on a 0.7% agarose gel

Gel extract OC-EGFP DNA fragment gel slice with QIAquick gel extraction kit

Purify OC-EGFP DNA fragment using Elutip Column

Generate and Analyze OC-EGFP Transgenic Mice
Figure 7. OC-EGFP Transgenic Offspring were Phenotyped by Identification of Green FluorescentVertebrae Tails. The mouse tail vertebra on the right is from line “F” OC-EGFP transgenic while the vertebra on the left is from a non-transgenic littermate. The ability to see the green fluorescence by microscopy eliminates the need for genotyping and allows for rapid phenotyping of the snipped tails. Magnification = 20X.
WT

OC-EGFP
Figure 8. EGFP Is Specifically Expressed in the Osteoblast Lineage in
Cryosections of 28 Day Old Transgenic Mice. Tissues were removed from
sacrificed mice and fixed in 4% paraformaldehyde. Fixed tissues were mounted,
frozen, cryosectioned in 5 μm increments and visualized by fluorescence microscopy.
GFP is expressed in osteoblasts and osteocytes in the tibia, calvarium and femur, but
not in the kidney of 28 day old line “F” transgenic mice (left and middle panels). No
expression was observed in either the femur or kidney of non-transgenic littermates
(right panels). Magnification = 400X for tibia section and 200X for all other sections.
Figure 9. EGFP Is Expressed in Differentiated Osteoblast Cells Derived from Bone Marrow Cultures of OC-EGFP Transgenic Mice. Mouse femur and tibia bone marrow from 8 week old transgenic mice was extracted and plated in 6-well dishes at a density of 3 x 10^6 cells/well. Osteoblast cells were differentiated using L-ascorbate and sodium inorganic phosphate. Bone marrow cultures on Day 12 (left) show that multilayered cells are starting to form (Visible) and show a few weakly fluorescent cells by fluorescence microscopy (EGFP). By Day 14 (middle), multilayered bone nodules are visible and EGFP expressing cells are readily observed by fluorescence microscopy in bone nodules. By the late stages of differentiation (Day 20, right), GFP is still detectable in osteoblasts in mineralized nodules but its expression level appears to be decreased. No GFP was observed in cells located in between the nodules.
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from calvaria osteoblasts derived from OC-EGFP mice (Owen et al., 2001). The results show that EGFP and endogenous OC were expressed concurrently during osteoblast differentiation. This result suggests that the OC-EGFP transgene is properly expressing EGFP during OB differentiation. Bone marrow cultures from 8-week old mice were extracted from OC-EGFP mice and placed under conditions for osteoblast differentiation (Figure 9). Expression of EGFP protein was visualized by fluorescence microscopy. The results show that EGFP protein was induced at day 12 in culture, which correlates with the onset of multilayered nodule formation (Figure 9). Moreover, EGFP was highly expressed in differentiated multilayered mineralized nodules at 14 and 20 days in culture (Figure 9), which correlates with the appearance of bone nodules and endogenous OC gene expression in matrix maturation and mineralization stages of osteoblast differentiation (Owen et al., 2001; Lian et al., 2001). EGFP is specifically expressed from the OC promoter in osseous, but not non-osseous cells in transgenic mice from embryonic day 17.5 to 1 year old (Owen et al., 2001). Taken together, these results suggest that the OC-EGFP transgenic mouse line “F” is a good model to study growth and differentiation of bone development.
DISCUSSION

In this study, we demonstrate that enhanced green fluorescent protein (EGFP) is expressed under the control of the osteocalcin (OC) promoter specifically in osteoblastic cells in vitro and is induced by 1,25 (OH)₂ vitamin D₃. Furthermore, OC-EGFP transgenic mice were generate and were shown to express EGFP specifically in osteoblasts and osteocytes in bone tissues. Genotyping of transgenic mice expressing EGFP was not necessary, since the EGFP expressing mice were directly phenotyped by observing the intrinsic green fluorescence of EGFP in the vertebrae of tail snips using fluorescence microscopy. Bone marrow extracted from OC-EGFP transgenic mice that were treated ex vivo to differentiate osteoprogenitor cells into osteoblasts show EGFP induction when bone nodules begin to form and endogenous OC is induced (Day 12-14 of culture). The expression EGFP under control of the OC promoter is consistent with previous results of the expression of endogenous OC and the formation of bone nodules with ex vivo cultures (Lian et al., 2001). It has been suggested that EGFP may be toxic to cells, however expression of the EGFP protein does not appear to have any toxic effects on the OC-EGFP transgenic mice. These mice are viable and express EGFP for at least 1 year after birth.

The OC-EGFP transgenic mice could be used to study regulation of OC by RUNX in vivo and to characterize the effect of potential new drugs on bone formation and resorption. These drugs may be utilized in therapeutic treatment of bone diseases such as osteoporosis and osteopenia. Osteoporosis and osteopenia are
bone diseases caused by a decrease in bone mass due to an increase in bone resorption and a decrease in bone formation. Patients with osteoporosis and osteopenia are subjective to multiple bone fractures. Osteoclastic bone resorption and osteoblastic bone formation are coupled processes that are influenced by many factors, which could contribute to bone diseases. Bone loss is brought about by an imbalance between bone resorption and bone formation (Rodan et al., 2002).

Therapies for osteoporosis to date involve drugs that inhibit bone resorption (Russell et al., 2001). However, future drug therapies could involve drugs that activate and recruit osteoblasts to form bone. High throughput screening assays could be used to develop these potential drugs to treat osteoporosis and osteopenia. Our results show that osteoblasts could be visualized easily using fluorescence microscopy in OC-EGFP transgenic mice. The recruitment of OBs expressing EGFP in response to treatment with a potential drug could be observed in OC-EGFP transgenic mice in vivo. It may be possible that a drug, which regulates RUNX expression levels and subsequently regulates bone phenotypic markers, such as OC may increase bone formation and be important for the treatment of osteoporosis and osteopenia. OC-EGFP mice may be used to study the effects of potential drugs on the regulation of OC by RUNX proteins.

Taken together, these findings suggest that the OC-EGFP transgenic mice are a good model for determining the effects of possible therapeutic drugs for the treatment of bone diseases and the in vivo function of RUNX-mediated regulation of OC.
Model for the Transient Association of RUNX Transcription Factors with Subnuclear Sites

In this study, our key findings are that RUNX transcription factors dynamically associate with stationary subnuclear domains in a C-terminal dependent mechanism in living cells (Chapter 1). Furthermore, two point mutations in the NMTS that decrease nuclear matrix association have no measurable effect on the subnuclear distribution and mobility of RUNX2 in living cells (Chapter 2). Moreover, RUNX1 and RUNX2 are targeted to common subnuclear domains (Chapter 1). The RUNX partner protein, CBFβ, is also targeted to these subnuclear foci (Chapter 1), which appear to represent sites of active transcription, although their relationship to individual genes or gene organization remains to be resolved (Chapter 1). In addition, OC-EGFP transgenic mice were generated, which create a useful tool for studying the in vivo function of RUNX-mediated osteocalcin regulation and for developing new drugs for treatment of bone diseases.

RUNX proteins are associated with the nuclear matrix at subnuclear foci through the NMTS (Zeng et al., 1997). The association of RUNX transcription factors with the nuclear matrix at the subnuclear domains suggests a link between gene expression and nuclear architecture (Stein et al., 1998; 2000b). The nucleus is compartmentalized into functional cellular substructures and several nuclear components involved in gene expression, DNA replication and splicing have been shown to reside in different subnuclear compartments (Nickerson et al., 1995; Stein et al., 2000b; Lamond and Earnshaw, 1998; Cook, 1999; Berezney et al., 2000; Misteli and Spector, 1998; Dundr and Misteli, 2001). However, little is known about how
these cellular processes are integrated into the nuclear architecture and their spatial and temporal organization in the three-dimensional nucleus (Dundr and Misteli, 2001). This study provides insight into the spatial and temporal localization and mobility of RUNX transcription factors within the nucleus and suggests a relationship between nuclear architecture and gene expression.

We show in Chapter 1 that RUNX factors localize to nuclear matrix-associated subnuclear foci and that a majority (70-80%) of RUNX subnuclear foci are associated with sites of transcription, which is consistent with previous results and results obtained concurrently with these studies (Zeng et al., 1997; 1998; Zaidi et al., 2001; Tang et al., 1999). RUNX subnuclear foci may contain transcription factor complexes, since co-regulatory proteins and nascent transcripts localize to RUNX sites (Chapter 1; Zaidi et al., 2002; Javed et al., 2000; Kundu et al., 2002).

Furthermore, RUNX subnuclear sites that are not associated with transcription, may be sites of storage or transcriptional repression. The assembly of RUNX proteins into multi-subunit transcription factor complexes appears to be very dynamic since RUNX proteins are highly mobile (half-time of recovery = 6-10 seconds) (Chapter 1). However, about 20-35% of RUNX proteins are immobilized in the nucleus possibly by interacting with the nuclear architecture. How transcription complexes assemble onto genes is an emerging question. Recently, in vivo kinetic modeling of RNA Polymerase (Pol) I transcription of the ribosomal RNA genes, which are located in the nucleolus, showed that the RNA Pol I machinery is highly dynamic (Dundr et al., 2002). RNA Pol I subunits enter the nucleolus as separate subunits and rapidly exchange between the nucleoplasm and ribosomal transcription sites. Assembly of
the RNA Pol I machinery is highly inefficient in vivo with the rate-limiting step assumed to be the successful formation of the elongation complex (Dundr et al., 2002). These results suggest that localization of the subunits to the RNA Pol I transcription complex at the transcription site is random, that efficient formation of multi-subunit complexes is very low and that the RNA Pol I subunits move by diffusion.

In contrast to the interpretation that nuclear proteins, such as the Pol I subunits and alternative splicing factor, ASF, move in a diffusional mechanism (Phair and Misteli, 2000; Dundr and Misteli, 2001; Dundr et al., 2002), our results suggest that RUNX factors may move in a directional mechanism targeted by the association of the C-terminus with the nuclear matrix and/or co-regulatory proteins. This is consistent with the conclusions that nuclear proteins may be constrained by the nuclear architecture, such as the nuclear matrix (Kruhlak et al., 2000; Stenoien et al., 2001). The high percent immobile fraction obtained for RUNX1 and RUNX2 (20-35%) suggests that a portion of RUNX proteins is immobilized by association with the nuclear architecture or within foci in a C-terminal dependent mechanism (Chapter 1 and 2). We show that RUNX proteins are targeted to subnuclear foci through the C-terminal domain. RUNX proteins constantly flow into and out of RUNX foci. A rapid exchange of proteins between nuclear compartments and the nucleoplasm has been shown for several nuclear compartments, which are in constant flux (Dundr and Misteli, 2001). We show that RUNX proteins also rapidly exchange between RUNX subnuclear foci and the nucleoplasmic pool.

The model from our studies shows a transient association of RUNX
transcription factors with subnuclear sites, of which a subset colocalize with sites of active transcription and co-regulatory factors. Therefore, the subnuclear foci may contain transcription factor complexes (Figure 1). The RUNX subnuclear foci are positionally stabilized in the nucleus by association with a structural component or perhaps a specific acceptor protein in the nuclear matrix. However, the association of RUNX factors with subnuclear foci is highly dynamic, reflecting a rapid exchange of transcription factors with the nucleoplasmic pool. Moreover, a fraction (20-35%) of RUNX factors are immobilized at subnuclear foci through the C-terminus. The mobility of RUNX transcription factors may be reduced though the association of the RUNX C-terminus with the nuclear matrix and/or co-regulatory proteins. The NMTS in the C-terminus of RUNX factors is important for targeting RUNX factors to the nuclear matrix and to sites of transcription (Chapter 1; Zeng et al., 1997; 1998). We propose that RUNX transcription factors dynamically assemble into transcription factor complexes and transiently reside in subnuclear domains for short periods of time. However, the R383 and Y413 amino acids that were mutated in the NMTS were not responsible for constraining the mobility since they had no measurable effect on the mobility of RUNX factors (Chapter 2). Therefore, either the entire NMTS or co-regulatory protein interaction domains in the C-terminus may be necessary to constrain the mobility of RUNX proteins. Furthermore, the targeting of RUNX factors to the nuclear matrix may involve an interaction with common acceptor protein(s), as RUNX factors contain a common intranuclear targeting signal and we show that RUNX1 and RUNX2 are targeted to common subnuclear domains.
Figure 1. A Model for the Transient Association of Transcription Factors with Subnuclear Protein Complexes. RUNX subnuclear foci (aqua and pink ovals) are nuclear matrix associated. Aqua ovals represent RUNX associated subnuclear transcription factor complexes and pink ovals represent RUNX storage sites. The nuclear matrix is represented by the black hatch marks. The pool of nucleoplasmic RUNX transcription factors (green background) is rapidly moving. RUNX subnuclear foci are positionally stabilized within the nuclear space. The limited movement of the subnuclear foci is shown by the black arrowheads in the nucleus of the whole cell (left). An area of the nucleus, represented by the purple box, is enlarged on the right and shows multi-subunit transcription complexes (aqua ovals (RUNX subnuclear foci) with pink circles (RUNX proteins) and purple and black circles (RUNX associated co-regulatory proteins)). RUNX proteins (pink circles) dynamically associate with RUNX subnuclear foci (arrows) through a C-terminal dependent mechanism by interacting with the nuclear matrix and/or co-regulatory proteins. The mobility of RUNX transcription factors is slowed down by this interaction. The mobility of RUNX into and out of storage sites is indistinguishable from transcription factor complexes by FRAP experiments.
Model: Transient Association of Transcription Factors with Subnuclear Protein Complexes

Subnuclear Foci Are Positionally Stabilized within the Nuclear Space on the Nuclear Matrix

Transcription Factors Transiently Associate with Subnuclear Foci

Subnuclear Foci May Represent Multi-subunit Transcription Factor Complexes

Mobility of Transcription Factors Are Slowed Down by Association with Nuclear Matrix and/or Interacting Proteins

- Pool of nuclear proteins
- Nuclear Matrix
- RUNX Transcription Factor
- Subnuclear co-regulatory complexes
- RUNX Storage Sites
(Chapter 1). However, other interpretations from this result may be that multiple RUNX proteins accumulate at a site containing a single acceptor protein by creating multiple interactions with several co-regulatory proteins localized at that focus. Alternatively, the acceptor protein(s) that may have its own recognition motif(s) for RUNX and for the nuclear matrix may serve as an organizing center for transcription factor complexes.

**Dynamics of Nuclear Proteins**

Recent studies here and from other laboratories using GFP-tagged nuclear proteins uncovered the highly dynamic nature of the nucleus (Chapters 1 and 2; Phair and Misteli, 2000; Kruhlak et al., 2000; Stenoien et al., 2000a; Stenoien et al., 2001). Alternative splicing factor (ASF) fused to GFP has a diffusion coefficient (D) of 0.24 μm/s² and was demonstrated to be approximately 100 times slower than GFP alone (Kruhlak et al., 2000; Phair and Misteli, 2000). Similar results were obtained for the mobility of ASF-GFP fusion protein in two individual studies (Phair and Misteli, 2000; Kruhlak et al., 2000), but they differed in their conclusions (Shopland and Lawrence, 2000). One interpretation was that the mobility of ASF-GFP is consistent with free diffusion and that the ASF proteins do not associate with any structural component (Phair and Misteli, 2000). Alternatively, the reduced movement of ASF-GFP proteins compared to GFP alone is consistent with a transient association with structural sites such as the nuclear matrix (Kruhlak et al., 2000). The question of whether ASF proteins are slowed down by association with nuclear architecture has yet to be determined (Shopland and Lawrence, 2000). In Chapter 1, we show that the
mobility of RUNX nuclear proteins is constrained by the C-terminal domain, which contains the NMTS and several co-regulatory protein interaction domains, suggesting that association with the nuclear architecture and/or co-regulatory proteins is important for reducing the mobility of RUNX transcription factors.

Nuclear matrix association of transcription factors in living cells has been demonstrated using the estrogen receptor α fused to GFP (ERα-GFP). ERα-GFP has a diffuse nuclear distribution in the absence of estrogen. In the presence of ligand, ERα becomes associated to the nuclear matrix in subnuclear sites (Stenoien et al., 2000a). Using FRAP analysis, it was shown that unligated ERα is highly mobile (half-time of recovery < 1 second). Treatment with estrogen decreases the mobility of ERα to a half-time of 5-6 seconds, which correlates with increased association with the nuclear matrix. Treatment with proteasome inhibitors or an estrogen antagonist immobilizes ERα to the nuclear matrix (half-time= 5-20 minutes) (Stenoien et al., 2001). These results suggest that association with the nuclear architecture retards the movement of nuclear factors, which supports our data from Chapter 1 that the C-terminus of RUNX proteins which contains the NMTS and co-regulatory factor domains reduces the mobility of RUNX proteins.

Cellular and Physiological Significance

Our results show that the intranuclear trafficking of RUNX transcription factors, which are necessary for development of hematopoietic and osteogenic tissues, is dynamic. RUNX factors move into and out of sites of transcription within seconds and appear to be constrained by association with the nuclear architecture.
Compartmentalization of the nucleus appears to be important for cellular processes such as gene expression, replication and splicing. We find that compartmentalization of RUNX transcription factors into subnuclear foci may have functional significance for gene expression, since RUNX foci contain nascent transcripts as shown by BrUTP incorporation and co-regulatory proteins. Our results provide insight into how RUNX factors move and are targeted to sites of transcription, which is a necessary cellular function.

One limitation of overexpressing RUNX proteins in the in situ immunofluorescence studies is that it is not known whether every RUNX focus is formed at a specific gene. One indication is that active transcription is occurring at 70-80% of RUNX foci, which suggests that a majority of the foci are localized to specific genes. However, these experiments expose new questions such as: Is only one gene localized to each focus or are there many genes? How many of the genes are active in each focus? Are proteins specifically associating with genes or other nuclear proteins at subnuclear foci? Answers to these questions may be important to evaluate in the future.

RUNX proteins are important transcription factors, which are required for tissue-specific development. The genes regulated by RUNX factors are necessary for differentiation and development of bone and hematopoietic tissues. The roles of RUNX genes in development are demonstrated by gene ablation studies in mice and by the human diseases that occur from mutations in these genes. Knockout of RUNX2 proteins or deletion of the C-terminus of RUNX2 results in loss of bone formation in mice. In addition, bone-specific genes, such as osteocalcin, are not
activated by RUNX2. Thus, RUNX2 deficiency causes a defect in osteoblast maturation (Komori et al., 1997; Otto et al., 1997; Choi et al., 2001). Mutations in RUNX2 have also been implicated in human bone diseases, such as cleidocranial dysplasia (CCD), suggesting a biological requirement for RUNX2 proteins in bone development at post-natal stages (Mundlos et al., 1997; Zhang et al., 2000). RUNX1 is necessary for hematopoietic development in RUNX1 deficient mice, demonstrating that the hematopoietic genes activated by RUNX1 during hematopoietic differentiation are not properly regulated (Wang et al., 1996a; Okuda et al., 1996; North et al., 1999). Chromosomal translocations of the RUNX1 gene result in human acute leukemias (Speck and Stacy, 1995). Furthermore, the C-termini of RUNX1 and RUNX2 were demonstrated to be necessary for hematopoiesis and skeletal development, respectively, since C-terminal truncated proteins produce similar phenotypes as RUNX1 and RUNX2 null mice (North et al., 1999; Choi et al., 2001). Thus, these results indicate the physiological importance of RUNX proteins and their C-terminal segments in tissue-specific development.

The third chapter lays the foundation to study the physiological events directly in an in vivo context. This chapter includes work demonstrating a new tool for the study of RUNX-regulated osteocalcin (OC) gene expression during osteoblast differentiation. We generated transgenic mice expressing EGFP under control of the OC promoter. Our results in vitro show that EGFP protein is specifically expressed in an osteoblastic cell line and that EGFP mRNA and protein are induced by vitamin D3. Furthermore, the OC-EGFP transgenic mice express EGFP specifically in the osteoblast lineage. Moreover, EGFP is expressed in mineralized bone nodules in
osteoblast differentiated bone marrow cells derived from transgenic mice. These results suggest that the OC-EGFP transgenic mice provide a good model for studying RUNX-mediated osteocalcin regulation.

Taken together, our findings presented in this thesis show that in living cells RUNX transcription factors dynamically associate with stationary subnuclear foci, which represent sites of active transcription, in a C-terminal dependent mechanism to regulate gene expression. Moreover, RUNX1 and RUNX2 are targeted to common subnuclear domains, reflecting the similarities in their subnuclear targeting signal. Furthermore, RUNX subnuclear foci appear to represent sites of transcription, containing co-regulatory proteins and nascent transcripts. These conclusions provide a mechanism for how RUNX transcription factors may associate with subnuclear foci to regulate gene expression. In addition, OC-EGFP mice provide a useful tool to develop drugs for treating bone diseases and studying the in vivo effects of RUNX-mediated osteocalcin regulation.
APPENDIX A

Saturability of Nuclear Matrix Associated RUNX Sites
INTRODUCTION

Factors involved in cellular processes including replication, transcription and mRNA splicing are associated with the nuclear scaffold at specific sites within the nucleus (Nickerson et al., 1995; Stein et al., 2000b; Lamond and Earnshaw, 1998; Cook, 1999; Berezney et al., 2000; Misteli and Spector, 1998). Nuclear compartmentalization may be maintained at least in part by association with the nuclear matrix (Stein et al., 2000b). The nuclear matrix consists of a network of highly branched 10-nm filaments and is comprised of insoluble proteins, hnRNA and DNA that is associated at matrix attachment regions (MARs) (Fey et al., 1984; 1986; Davie, 1997; Nickerson et al., 1995). Several nuclear matrix associated proteins have been discovered including B23 and hnRNPs, which are involved with RNA metabolism, Lamins, NuMa, PML and RUNX (Zeng et al., 1997; Zeng et al., 1994; Zaidi et al., 2001; Tang et al., 1999; Stein et al., 2000b; Chang et al., 1995; Barboro et al., 2002; Mattern et al., 1996; Goldman et al., 2002). RUNX proteins are associated with the nuclear matrix in a punctate distribution (Zeng et al., 1997; Zaidi et al., 2001; Tang et al., 1999). To date, none of the known nuclear matrix associated proteins has displayed a filament-like staining pattern by immunohistochemical studies (Nickerson et al., 1995; Stein et al., 2000b). These findings suggest that the nuclear matrix may consist of multiple protein complexes interacting with each other in a manner that produces the 10-nm intermediate filament structures; however when only one protein is observed at a time it appears punctate in nature.

Since RUNX1 and RUNX2 are targeted to common subnuclear domains (Chapter 1), we hypothesize that RUNX proteins bind to a specific acceptor protein at
nuclear matrix-associated docking sites. One approach to assess the validity of this working model is to examine whether RUNX sites are saturable and whether non-physiological sites or compartments can be occupied after forced expression of RUNX proteins.

In this study, we find with increasing protein concentration the RUNX foci increase in size, but the number of foci slightly decreases using in situ fluorescence microscopy. Furthermore, RUNX proteins do not appear to compete with high concentrations of competitor for nuclear matrix sites, which suggest that RUNX foci may not be saturable. However, localization of RUNX in the cytoplasm by in situ immunofluorescence and extraction of RUNX in non-physiological compartments in the biochemical fractionation assays at high concentrations of protein suggests that RUNX sites may be saturable. The results obtained produced opposing conclusions as to whether RUNX sites are saturable and therefore, were not able to be interpreted. However, the results may reflect a gradual accumulation of RUNX proteins at subnuclear foci.
MATERIALS AND METHODS

_In situ_ Immunofluorescence

SaOS-2 cells were grown on 0.5% (w/v) gelatin-coated coverslips in 6-well plates and cultured until 70% confluent. Cells were transiently transected using the LipofectAMINE Plus method (Life Technologies, Grand Island, NY) with increasing concentrations (0.25, 0.5 or 1 μg) of expression plasmids maintaining a total DNA concentration of 1 μg using empty vector. Whole cell (WC), cytoskeleton (CSK) and nuclear matrix-intermediate filament (NMIF) preparations were performed and fluorescence images were captured as previously described (Chapter 1, Materials and Methods). Cells were counted for level of expression and for whether the subcellular distribution was cytoplasmic and nuclear or only nuclear. To calculate the number and the size of the foci, 10-13 planes of Z-series images were captured in 0.5 μm increments. The stack of images was combined into one image using the average Stack Arithmetic function and the out of focus light was removed with the 2D deconvolution function using the nearest neighbors function in MetaMorph. Deconvoluted images were thresholded and counts of the number and size of the foci were determined using the Integrated Morphometry Analysis drop-in. The integrated intensity of the nuclei before deconvolution was determined by selecting the nuclei with the polygon region tool and using the Region Measurements function to calculate the intensity of the selected region.
Biochemical Subcellular Fractionation

HeLa cells were plated at 1.0 x 10^6 cells per 100 mm plate and transfected using Superfect at 80% confluence with 1 μg EGFP-RUNX2 and increasing concentrations (0, 8, 16 μg) of untagged-RUNX2 maintaining a total DNA concentration of 17 μg with empty vector. Cells were harvested 12-20 hours following transfection. Cells were washed twice with PBS, scraped in 1 ml of PBS and placed in a 1.5 ml microcentrifuge tube. Plates were washed once with 500 μl of PBS and the 1.5 ml of cells were centrifuged at 3600 rpm (~880 g) for 5 minutes. The supernatant was aspirated and the pellets were resuspended in 300 μl of mammalian lysis buffer (8 M Urea, 0.1 M NaH2PO4, 0.1 M Tris-HCl pH 8.0, 1X Complete Protease Inhibitor, 25 mM MG132 proteosome inhibitor) for whole cell fractions (Flowchart A). For CSK, DNase and NMIF fractions, a second plate was harvested as above and the pellet was extracted twice on ice for 15 minutes each with 150 μl CSK buffer (100 mM NaCl, 0.3 M sucrose, 10 mM pipes, pH 6.8, 3 mM MgCl2, 1 mM EGTA, 0.5% Triton X-100, 2 mM Vanadyl Ribonucleoside Complex (VRC), 1X Complete Protease Inhibitor, 25 mM MG132 proteosome inhibitor). Following each extraction the cells were centrifuged for 5 minutes at 3600 rpm and the supernatants were pooled as the CSK fraction. The pellets were digested twice for 30 minutes each in 150 μl of digestion buffer (CSK buffer with 50 mM NaCl) with 400 Units/ml of RNase-free DNase I (Roche, Indianapolis, IN) rocking at room temperature. The cells were centrifuged for 5 minutes at 3600 rpm. Following the second 30 minutes digestion, DNA and soluble proteins were extracted with 0.25 M ammonium sulfate on ice for 10 minutes and centrifuged for 5 minutes at 3600 rpm.
Flowchart A. Schematic of Biochemical Subcellular Fractionation Method. Two plates were transfected, one plate for the whole cell (WC) fraction and the other plate for the sequential extractions of the cytoskeleton (CSK), chromatin (DNase) and nuclear matrix-intermediate filament (NMIF) fractions. The plates were scraped in PBS, centrifuged at 3,600 rpm for 5 minutes. The WC pellet was lysed in mammalian lysis buffer for 5 minutes on ice. The cell pellet from the second plate was extracted with CSK buffer twice to remove soluble proteins and the supernatants after centrifugation were pooled as the CSK fraction. The pellet after the second CSK extraction was digested with DNase I twice and then extracted with 0.25 M ammonium sulfate to remove chromatin. The supernatants after centrifugation were pooled as the DNase fraction and the cell pellet was dissolved in UMN buffer as the NMIF fraction.
Biochemical Subcellular Fractionation Methods

Morphology

1. Intact Cell

- Protocol: Lysis buffer
  (8 M Urea, 0.1 M Na₂HPO₄, 0.1 M Tris-Cl pH 8.0)

- Protein fraction: Whole cell Proteins (WC)

2. Intact Cell

- Protocol: CSK buffer (2 X 15 min)
  (high salt conc.)

- Protein fraction: Soluble Proteins (CSK)

Cytoskeletal framework

- Protocol: DNase I (2 X 30 min)
  then 250 mM ammonium sulfate (10 min)

- Protein fraction: Chromatin (DNase)

Nuclear matrix-intermediate filament scaffold

- Protein fraction: Nuclear matrix-intermediate filaments (NMIF)
The supernatants were pooled as the DNase fraction. The pellets (NMIF fraction) were resuspended in 300 μl of UMN buffer (8 M Urea, 2% β-mercaptoethanol, 2% NP-40, 1X Complete Protease Inhibitor, 25 mM MG132 proteosome inhibitor).

Equivalent cell numbers for each fraction were loaded on an 8% polyacrylamide gel. Proteins were detected using a mouse monoclonal RUNX2/Cbfal antibody (a gift from Y. Ito, National University of Singapore; 1:2,000 dilution) and an HRP-conjugated α-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2,000 dilution).
RESULTS

Saturability of Nuclear Matrix-Associated RUNX Sites

Previous results in cell-free systems have shown that the estrogen receptor and crude, but not partially purified, dexamethasone receptor complexes bind to the nuclear matrix with high affinity and that the binding sites are saturable (Satoh et al., 1986; Metzger et al., 1990). To assess whether the nuclear matrix-associated RUNX sites are saturable within a cellular context, we performed both biochemical subcellular fractionation and in situ immunofluorescence assays. For the biochemical subcellular fractionation studies, our strategy was to overexpress a constant concentration (1 µg) of EGFP-RUNX2 and determine whether the nuclear matrix association of EGFP-RUNX2 could be competed by overexpressing increasing concentrations (0, 8 and 16 µg DNA) of untagged-RUNX2 protein in HeLa cells in 100 mm plates, which do not contain endogenous RUNX proteins. If RUNX sites were saturable, then we would expect that as the concentration of untagged-RUNX2 increases, association of EGFP-RUNX2 with the nuclear matrix will decrease and EGFP-RUNX2 will be displaced to either the DNase or CSK fraction since untagged-RUNX2 will compete with EGFP-tagged RUNX2 for the nuclear matrix docking sites. Calculations combined from four experiments show that as the concentration of untagged-RUNX2 increases, relative levels of EGFP-RUNX2 in the CSK and DNase fractions decrease, and in the NMIF fraction increase (Figure 1B). This result suggests that the RUNX sites may not be saturable. However, EGFP-RUNX2 and RUNX2 are extracted in the CSK and DNase fractions at higher concentration of
competitor, which shows that EGFP-RUNX proteins may be displaced by the competitor into the soluble and chromatin fractions by competing for nuclear matrix sites (Figure 1A and C). This result suggests that RUNX sites may be saturable and that RUNX may be occupying non-physiological compartments at higher concentrations of the proteins. The interpretation of our results was confounded by the observation that the expression of RUNX2 was not linear with the DNA concentration and appeared to influence EGFP-RUNX2 levels. The amount of EGFP-RUNX2 associated with the nuclear matrix was shown to increase as the concentration of untagged-RUNX2 increased (Figure 1A and C). It is likely that untagged-RUNX2 may influence the expression levels of EGFP-RUNX2 by associating with a RUNX consensus binding site at nucleotides 619-625 in the complementary strand of the CMV promoter in the pcDNA3 vector (Invitrogen) into which EGFP-RUNX2 was cloned. Thus, the experimental limitations of this approach have confounded the interpretation of the results.

In another strategy to determine whether RUNX sites are saturable, we performed in situ fluorescence microscopy with SaOS-2 cells transfected in 6-well plates with increasing concentrations (0.25, 0.5 and 1.0 μg) of expression vectors encoding EGFP-RUNX fusion proteins or EGFP alone and performed WC, CSK and NMIF preparations. The total DNA concentration was maintained at 1 μg using empty vector. Because RUNX proteins are nuclear proteins, we would expect that cytoplasmic localization of EGFP-RUNX proteins may represent occupancy of a non-physiological compartment. Therefore, the number of cells expressing EGFP-RUNX fusion proteins either in the nucleus only or in the cytoplasm and nucleus...
Figure 1. Nuclear Matrix-Associated EGFP-RUNX2 Is Not Competed by Increasing Concentrations of Untagged-RUNX2. HeLa cells were transiently transfected in 100 mm plates with a constant amount (1 μg) of EGFP-RUNX2 expression plasmid and increasing concentrations (0, 8 and 16 μg) of untagged-RUNX2 maintaining the total DNA concentration at 17 μg. Biochemical WC, CSK, DNase and NMIF fractions were performed as described in Flowchart A and the Materials and Methods. Equal cell equivalents were loaded on the gel for each fraction. (A) Western blot analysis was performed using a mouse monoclonal RUNX2 antibody (Y. Ito, Japan; 1:2,000 dilution) to detect both EGFP-RUNX2 and untagged RUNX2 proteins on the same membrane. A lighter exposure of the untagged-RUNX2 bands is shown under the western blot. (B and C) The membrane was exposed to the Storm 840 Phosphoimager (Molecular Dynamics, Sunnyvale, CA) while emitting chemiluminescence light to quantitate the bands. An average from four experiments of the phosphoimager values are shown in (C) for each fraction. (B) The average of the WC fractions were normalized to 1 by dividing by themselves since the amount of protein in the WC fraction should approximately equal the total amount of protein in the CSK + DNase + NMIF fractions. The CSK, DNase and NMIF fractions were divided by the total amount of protein all three fractions (C+D+N= CSK + DNase + NMIF) to show the relative amount of protein in each of the fractions at each DNA concentration. Error bars represent standard deviations (S.D.).
A

1 μg EGFP-RUNX2

<table>
<thead>
<tr>
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<th>WC</th>
<th>CSK</th>
<th>DNase</th>
<th>NMIF</th>
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<td>8</td>
<td>16</td>
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EGFP-RUNX2

RUNX2

Lighter exposure of RUNX2

B

C+D+N = CSK + DNase + NMIF

Average of each fraction

Total amount of protein in all fractions for that plate

0 μg
8 μg
16 μg

EGFP-RUNX2

RUNX2
C

[Graph showing arbitrary units for different samples: EGFP-RUNX2 and Untagged-RUNX2 with variations in DNase and NMIF concentrations.]

- 0 μg
- 8 μg
- 16 μg
were counted. The results for EGFP-RUNX1 (Table 1) and EGFP-RUNX2 (Table 2) are averages from two experiments (± S.D.). The results show that as the concentration of EGFP-RUNX1 (Figure 2A; Table 1) or EGFP-RUNX2 (Figure 3A; Table 2) increases a higher percentage of cells exhibit cytoplasmic staining in WC, CSK and NMIF preparations. Furthermore, the results demonstrate that the fluorescence intensity of the cells with cytoplasmic and nuclear staining increases with increasing concentrations of EGFP-RUNX1 (Figure 2B; Table 1) and EGFP-RUNX2 (Figure 3B; Table 2). In addition, as the DNA concentration increases, the percent of cells with dim nuclear expression decreases (Table 1 and Table 2). However, the cytoplasmic staining appears to be in the form of insoluble protein aggregates (Figures 4A and B; shown in the 1 µg image, but are also observed to a lesser extent in the 0.25 µg and 0.5 µg transfected cells). The aggregates were also observed in the CSK and NMIF extracted cells transfected with EGFP-RUNX2Δ361 (Figure 5A; Table 3) and EGFP alone (Figure 5B; Table 4). These results suggest that RUNX sites may be saturable. However, the increase of the cytoplasmic aggregates at 1 µg of protein makes it difficult to interpret these results since this insoluble aggregate may be an artifact from overexpression of the EGFP protein and may be caused by dimerization of the EGFP molecules or may localize in the cytoplasm because nuclear import was inhibited or nuclear export was increased.

In another approach to assess whether RUNX sites are saturable, we determined the number and size of the foci as the protein concentration increases. Z-series images of nuclei in NMIF preparations with different concentrations (0.25, 0.5
Figure 2. The Amount of EGFP-RUNX1 Protein in Cytoplasm Increases as the DNA Concentration Increases. SaOS-2 cells were transiently transfected in 6-well plates with increasing concentrations (0.25, 0.5 and 1.0 µg) of EGFP-RUNX1 expression plasmid. WC, CSK and NMIF preparations were performed on coverslips and the cells were counted using fluorescence microscopy for Bright, Medium and Dim expressing cells in either the nucleus only or the cytoplasm and nucleus (see Table 1). (A) The percent of bright, medium and dim expressing cells were combined for nuclear only staining (blue bars) and for cytoplasmic and nuclear staining (red bars). (B) The percent of bright cytoplasmic and nuclear stained cells for each DNA concentration (yellow, 0.25 µg; red 0.5 µg; blue, 1.0 µg) and cell preparation were plotted. At least 100 transfected cells were counted per experiment in two experiments. The error bars represent S.D.
EGFP-RUNX1

A

\[ \text{% Cells} \]

\begin{array}{cccc}
\text{WC} & \text{CSK} & \text{NMIF} & \text{WC} \\
0.25 \mu g & & & 0.5 \mu g \\
\text{WC} & \text{CSK} & \text{NMIF} & \text{WC} \\
& & & 1 \mu g \\
\end{array}

B

\[ \text{% Bright Cytoplasmic and Nuclear Cells} \]

\begin{array}{ccc}
\text{WC} & \text{CSK} & \text{NMIF} \\
0.25 \mu g & & \\
0.5 \mu g & & \\
1 \mu g & & \\
\end{array}
Table 1. In Situ Immunofluorescence Analysis of SaOS-2 Cells Transfected with Increasing Concentrations of EGFP-RUNX1

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<th>Constructs</th>
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<th>Nuclear Medium</th>
<th>Nuclear Dim</th>
<th>Cyto &amp; Nucl Bright*</th>
<th>Cyto &amp; Nucl Med*</th>
<th>Cyto &amp; Nucl Dim</th>
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<td>EGFP-RUNX1 0.25 μg</td>
<td>WC</td>
<td>9.9 ± 4.1%</td>
<td>35.4 ± 15.0%</td>
<td>39.5 ± 7.8%</td>
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* Insoluble cytoplasmic aggregates are present in these cells

**Bold print**=significant differences
Figure 3. The Number of Cells with EGFP-RUNX2 Protein in Cytoplasm Increases as the DNA Concentration Increases. SaOS-2 cells were transiently transfected in 6-well plates with increasing concentrations (0.25, 0.5 and 1.0 μg) of EGFP-RUNX2 expression plasmid. WC, CSK and NMIF preparations were performed on coverslips and the cells were counted using fluorescence microscopy for Bright, Medium and Dim expressing cells in either the nucleus only or the cytoplasm and nucleus (see Table 2). (A) The percent of bright, medium and dim expressing cells were combined for nuclear only staining (blue bars) and for cytoplasmic and nuclear staining (red bars). (B) The percent of bright cytoplasmic and nuclear stained cells for each DNA concentration (yellow, 0.25 μg; red 0.5 μg; blue, 1.0 μg) and each cell preparation were graphed. At least 100 transfected cells were counted per experiment in two experiments. The error bars represent standard deviations.
EGFP-RUNX2

A

% Cells

Nuclear only
Cytoplasmic and Nuclear

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B

% Bright Cytoplasmic and Nuclear Cells

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Table 2. In Situ Immunofluorescence Analysis of SaOS-2 Cells Transfected with Increasing Concentrations of EGFP-RUNX2.

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<td>27.9 ± 12.5%</td>
<td>7.4 ± 2.0%</td>
<td>0.4 ± 0.5%</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>CSK</td>
<td>21.7 ± 1.9%</td>
<td>34.8 ± 4.0%</td>
<td>23.4 ± 6.5%</td>
<td>17.2 ± 4.5%</td>
<td>3.0 ± 0.0%</td>
<td>-</td>
<td>253</td>
</tr>
<tr>
<td></td>
<td>NMIF</td>
<td>18.6 ± 3.4%</td>
<td>25.3 ± 4.6%</td>
<td>28.0 ± 4.2%</td>
<td>23.5 ± 2.1%</td>
<td>5.2 ± 4.0%</td>
<td>-</td>
<td>224</td>
</tr>
</tbody>
</table>

* Insoluble cytoplasmic aggregates are present in these cells

Bold print=significant differences
Figure 4. Cytoplasmic Staining is Predominately in the Form of Insoluble Globular Aggregates. SaOS-2 cells were transiently transfected in 6-well plates with increasing concentrations (0.25, 0.5 and 1.0 μg) of (A) EGFP-RUNX1 and (B) EGFP-RUNX2. WC and NMIF preparations were performed on coverslips and the cells were imaged using fluorescence microscopy. Images were captured using the MetaMorph Imaging Software at 400 ms exposure times. Globular cytoplasmic aggregates are shown in 1.0 μg WC images however, were also observed at lower DNA concentrations.
Figure 5. Insoluble Globular Cytoplasmic Aggregates Appear to Be an Artifact from Overexpressing of EGFP. SaOS-2 cells were transiently transfected in 6-well plates with increasing concentrations (0.25, 0.5 and 1.0 µg) of (A) EGFP-RUNX2Δ361 and (B) EGFP expression plasmids. WC and NMIF preparations were performed and the cells were imaged using fluorescence microscopy with the MetaMorph Imaging Software at 400 ms exposure times. Globular aggregates in the cytoplasm are shown in 0.25, 0.5 and 1.0 µg NMIF images. EGFP-RUNX2Δ361 proteins were completely extracted in NMIF preparations at the 0.25 µg DNA concentration.
Table 3. In Situ Immunofluorescence Analysis of SaOS-2 Cells Transfected with Increasing Concentrations of EGFP-RUNX2Δ361

<table>
<thead>
<tr>
<th>Constructs</th>
<th>prep</th>
<th>Nuclear Bright</th>
<th>Nuclear Medium</th>
<th>Nuclear Dim</th>
<th>Cyto &amp; Nucl Bright</th>
<th>Cyto &amp; Nucl Med</th>
<th>Cyto &amp; Nucl Dim</th>
<th>Total # Cells Counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-RUNX2Δ361 0.25 µg</td>
<td>WC</td>
<td>-</td>
<td>1.6 ± 2.3%</td>
<td>15.4 ± 2.3%</td>
<td>14.6 ± 5.7%</td>
<td>35.1 ± 3.3%</td>
<td>33.4 ± 2.3%</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td>CSK</td>
<td>3 ± 4.2 cells with incomplete extraction, globular dots in cytoplasm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NMIF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>EGFP-RUNX2Δ361 0.5 µg</td>
<td>WC</td>
<td>-</td>
<td>0.5 ± 0.6%</td>
<td>4.5 ± 5.0%</td>
<td>21.4 ± 0.5%</td>
<td>41.3 ± 0.4%</td>
<td>32.5 ± 3.5%</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td>CSK</td>
<td>13.5 ± 12 cells with incomplete extraction, globular dots in cytoplasm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NMIF</td>
<td>9 ± 5.7 cells with incomplete extraction, globular dots in cytoplasm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFP-RUNX2Δ361 1 µg</td>
<td>WC</td>
<td>-</td>
<td>-</td>
<td>5.4 ± 7.6%</td>
<td>30.3 ± 1.7%</td>
<td>35.1 ± 0.1%</td>
<td>29.2 ± 9.5%</td>
<td>285</td>
</tr>
<tr>
<td></td>
<td>CSK</td>
<td>65 ± 7.1 cells with incomplete extraction, globular dots in cytoplasm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NMIF</td>
<td>46.5 ± 2.1 cells with incomplete extraction, globular dots in cytoplasm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4. In Situ Immunofluorescence Analysis of SaOS-2 Cells Transfected with Increasing Concentrations of EGFP Protein

<table>
<thead>
<tr>
<th>Constructs</th>
<th>prep</th>
<th>Nuclear Bright</th>
<th>Nuclear Medium</th>
<th>Nuclear Dim</th>
<th>Cyto &amp; Nucl Bright</th>
<th>Cyto &amp; Nucl Med</th>
<th>Cyto &amp; Nucl Dim</th>
<th>Total # Cells Counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP 0.25 μg</td>
<td>WC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>17%</td>
<td>48%</td>
<td>35%</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>CSK</td>
<td>51 cells with incomplete extraction, globular dots in cytoplasm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NMIF</td>
<td>48 cells with incomplete extraction, globular dots in cytoplasm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFP 0.5 μg</td>
<td>WC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10%</td>
<td>27%</td>
<td>63%</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>CSK</td>
<td>25 cells with incomplete extraction, globular dots in cytoplasm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NMIF</td>
<td>15 cells with incomplete extraction, globular dots in cytoplasm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFP 1 μg</td>
<td>WC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13%</td>
<td>43%</td>
<td>44%</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td>CSK</td>
<td>18 cells with incomplete extraction, globular dots in cytoplasm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NMIF</td>
<td>22 cells with incomplete extraction, globular dots in cytoplasm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
and 1 μg) of EGFP-RUNX2 constructs expressed were captured using MetaMorph. The stack of images was averaged into a single image and deconvoluted using MetaMorph (see Materials and Methods). The number and size of the foci were determined from the deconvoluted images and the integrated intensities of the nuclei were determined using the averaged single image before deconvolution. The results show that the mean integrated intensity of the nuclei significantly increased as the concentration of DNA increased (n=9-10 for each DNA concentration) (Figure 6A). However, the number of foci and the average size of the foci were not significantly different as the concentration of DNA increases in a random population of cells at each DNA concentration (n=9-10) (Figure 6B and C). The number of foci ranged from 49-149 foci per nucleus and the average size of the foci ranged from 0.2 to 0.53 microns (Figure 7A and B). Moreover, when the integrated intensity of each nucleus from all three concentrations (n=29) was plotted against the number of foci for that same nucleus, the number of foci slightly decreased as the intensity increased (Figure 7A). When a scatter plot of the integrated intensity of each nucleus from all three concentrations (n=29) was graphed against the average area of the foci for that nucleus, then the results show that as the intensity of EGFP-RUNX2 protein in each cell increases, the size of the foci increases (Figure 7B). Furthermore, as the size of the foci increases the number of foci per nucleus slightly decreases (Figure 7C). Taken together, these results suggest that the RUNX that RUNX factors may gradually accumulate at subnuclear foci.
Figure 6. The Average Intensity of the Nucleus Increases as the Concentration of DNA Increases. SaOS-2 cells were transiently transfected in 6-well plates with increasing concentrations (0.25, 0.5 and 1.0 μg) of EGFP-RUNX2 expression plasmid. Z-series images of 9 or 10 cells from NMIF preparations were captured taking 10-13 planes at 0.5 μm increments using 200 ms exposure times. (A) The Arithmetic Stack function in MetaMorph was used to produce a single image that the integrated intensities were determined from. The nucleus was outlined using the polygon tool and the integrated intensity was determined using the Region Measurement function. (B) The arithmetic stack was then deconvoluted using the 2D-deconvolution function with Nearest Neighbors. The deconvoluted images were thresholded and the number and size of the foci were automatically counted using the Integrated Morphometry Analysis function. The average (A) integrated intensity, (B) number of foci and (C) area of the foci were determined for each DNA concentration (n=10 for 0.25 and 0.5 μg; n=9 for 1.0 μg). The error bars represent the standard error of the mean (SEM). Statistical differences were only observed with the average integrated intensity between 0.25 and 1.0 μg of DNA (P<0.05) using multiple pair-wise comparisons in the Tukey’s studentized range test and the Bonferroni t-test.
Concentration of DNA (μg)

A. Average Integrated Intensity of the Nucleus (x10^6)

B. Average Number of Foci

C. Average Area of Foci (μm^2)
Figure 7. Scatter Plots for Each Individual Nucleus Show an Increase in the Size of the Foci as the Intensity of the Nucleus Increases. SaOS-2 cells were transfected and Z-series images were captured and processed as described in Figure 10. (A) The number of foci for each nucleus (n=29) was plotted against the integrated intensity for that nucleus. (B) The average area of the foci for each nucleus (n=29) was graphed against the integrated intensity for that nucleus. (C) The average area of the foci was plotted against the number of foci for each nucleus (n=29). (A, B and C) A trendline (Linear line) was graphed to determine the correlation of one variable to the other. The equation and the R² value of the lines are shown.
A

Number of Foci

Integrated Intensity (x10^-7)

- Each nucleus
- Linear

y = -25835x + 1E+07
R^2 = 0.002

B

Average area of foci (μm^2)

Integrated Intensity (x10^-7)

- Each nucleus
- Linear

y = 5E+07x - 6E+06
R^2 = 0.2128

C

Average area of Foci (μm^2)

Number of Foci per nucleus

- Each nucleus
- Linear

y = -27.54x + 95.091
R^2 = 0.02
DISCUSSION

Results in this chapter provide the initial findings of an approach to address whether RUNX proteins bind to a finite number of specific sites and therefore show saturable binding. Our results show with increasing protein concentration the size of the foci increases and the number of foci slightly decreases. These results suggest that RUNX proteins may gradually accumulate at nuclear matrix-associated subnuclear sites with increasing protein concentrations.

The copy number of plasmids that are transfected into each cell, which cannot be physically controlled, determined the amount of protein that was expressed. Therefore, each transfected cell had different amounts of protein expressed, which was observed as bright, medium and dim expressing cells. Although an overall increase in protein concentration was observed at higher DNA concentrations, the cells selected for the in situ immunofluorescence experiments were random. In this random population, no difference was observed for the number and size of the foci for each of the DNA concentrations as a population. However, on a cell-by-cell basis there was an increase in the size for the foci at higher protein concentrations, suggesting that the variation of DNA copy number and therefore the protein concentration in the cell appears to have had an effect on the size of the foci.

The findings from these initial investigations are summarized here and the technical limitations, which impact the interpretations, are discussed below. At higher nuclear protein concentrations of RUNX factors, the kinetics of the association/disassociation may favor the association of RUNX factors with the
subnuclear foci. This is suggested by our findings that the size of the foci is enlarged at increased protein concentrations. The limit to which the RUNX factors can associate with the subnuclear foci represents a measure of the saturability of the foci. We observed a gradual accumulation of RUNX factors at subnuclear foci, suggesting that the foci may not have reached the point of saturation. However, the possibility exists that RUNX sites may be saturable. This is supported by the result that EGFP-RUNX2 may be competed by untagged-RUNX2, since at high concentrations of competitor EGFP-RUNX2 is displaced into the soluble and chromatin fractions in biochemical subcellular fractionation assays. If RUNX sites are saturable, then this may suggest that RUNX proteins may specifically interact with acceptor proteins at subnuclear foci and that the number of specific interactions may be limited. However, if RUNX sites are not saturable, then this may suggest that RUNX factors may not specifically interact with an acceptor protein or that the number of acceptor proteins at the subnuclear foci is not rate limiting.

Future studies will be needed to address several technical limitations identified here. First, experimental limitations of the biochemical fractionation were produced from the confounding issues that the expression of EGFP-RUNX2 was not linear with the DNA concentration and the expression of untagged-RUNX2 appeared to influence EGFP-RUNX2 levels. Second, in the immunofluorescence microscopy studies, an insoluble aggregate was observed in the cytoplasm of EGFP-RUNX1 and EGFP-RUNX2, which was also observed with the C-terminal deleted RUNX2 and EGFP alone. Third, the 1 μg concentration in the immunofluorescence experiments may have been at a concentration that was expressing the proteins at much higher
than physiological levels. It is possible that the EGFP-RUNX fusion proteins may not have been able to enter the nucleus through inhibition of nuclear import or because the proteins dimerized through the EGFP molecule. Therefore, the proteins may have accumulated in the cytoplasm and aggregated.

These findings suggest that RUNX factors may gradually accumulate at subnuclear foci. However, further studies will be needed to conclude whether RUNX sites are saturable.
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