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NP/NMP4 Transcription Factors Have Distinct Osteoblast Nuclear Matrix Subdomains

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Abstract The mechanisms underlying the coupling of type I collagen and matrix metalloproteinase (MMP) expression to cell structure and adhesion are poorly understood. We propose that nuclear matrix architectural transcription factors link cell structure and transcription via their association with nuclear matrix subdomains and by their capacity for altering promoter geometry. NP/NMP4 are nuclear matrix proteins that contain from five to eight Cys_2His_2 zinc fingers. Some NP/NMP4 isoforms bind to the rat type I collagen α1(I) polypeptide chain promoter in the manner of architectural transcription factors and alter basal transcription in osteoblast-like cells (Thunyakitpisal et al. in review). Certain isoforms of NP/NMP4 are identical to CIZ, Cas-interacting zinc finger protein, a nucleocytoplasmic shuttling protein that associates with focal adhesions and regulates MMP expression [Nakamoto et al. (2000): Mol Cell Biol 20:1649–1658]. To better understand the role of subnuclear architecture in collagen and MMP expression, we mapped the osteoblast nuclear distribution of NP/NMP4 proteins and identified the functional motifs necessary for nuclear localization and nuclear matrix targeting. Immunofluorescence microscopy was used to determine the cellular and subnuclear distribution of native NP/NMP4 proteins and green fluorescent protein (GFP)-NP/NMP4 fusion proteins in osteoblast-like cells. All GFP-NP/NMP4 fusion proteins localized to the nucleus, but accumulated in distinct nuclear matrix subdomains. The zinc finger domain was necessary and sufficient for nuclear import and matrix targeting. We conclude that the arrangement of the NP/NMP4 zinc fingers largely determines the subnuclear location of these isoforms. J. Cell. Biochem. 79:506–517, 2000.

Key words: bone; architectural transcription factors; CIZ; zinc fingers collagen; matrix metalloproteinases

The molecular mechanics that couple type I collagen and matrix metalloproteinase (MMP) expression to cell structure and the adhesion of the cell to the extracellular matrix are poorly understood [Gardner et al., 1999; Esparza et al., 1999; Dhawan et al., 1991; Benya and Shaffer, 1982]. The tissue matrix, i.e., the connections between the extracellular matrix, integrins, the cytoskeleton, and the nuclear matrix [Pienta and Coffey, 1992] has been postulated to mediate the transfer of mechanical signals through cells [Maniotis et al., 1997; Ingber, 1997], but the nuclear events that transduce changes in tissue matrix organization into changes in gene expression have not been identified.

The nuclear spatial distribution of proteins that mediate the transmission of mechanical signals appears critical to their function [Lelièvre and Bissell, 1998]. During adhesion complex-mediated signaling, the nuclear translocation of structural signal transducers, such as zyxin or β-catenin, may direct the redistribution of nuclear matrix proteins to form transcriptional subdomains that underlie the cellular response to the mechanical signal [Lelièvre and Bissell, 1998]. Subdomains are dynamically variable structural compartments that perform specific metabolic functions such as

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splicing, transcription, and DNA replication [Lelièvre and Bissell, 1998; Maraldi et al., 1998; Pombo et al., 1998; Spector, 1993]. Depending on their function, subdomains exhibit speckled, diffuse, or foci patterns when viewed with immunofluorescence microscopy [Larsson et al., 1995; Spector, 1993; Spector et al., 1991].

The geometry of the target gene promoter may also be significant in mechanotransduction [Bidwell et al., 1998]. Upon shuttling to the nucleus, β-catenin interacts with the architectural transcription factors LEF-1/TCF [Eastman and Grosschedl, 1999]. These transacting proteins bend or loop the DNA in the assembly of multiprotein enhancer complexes along the promoter [Eastman and Grosschedl, 1999]. Therefore, a change in cell shape or adhesion may ultimately lead to a change in the shape of the gene [Bidwell et al., 1998].

We have proposed the concept of the nuclear matrix architectural transcription factor to convert changes in osteoblast structure into alterations in collagen transcription, by virtue of its association with the nuclear scaffold and its capacity for mediating changes in promoter geometry [Bidwell et al., 1998]. We hypothesize that an architectural transcription factor localized to the nuclear matrix could bridge the mechanical signal transfer between the cytoskeleton and the gene promoter [Bidwell et al., 1998]. The spatial localization or subdomains of these kinds of proteins would act to "hardwire" the response of the collagen gene to specific changes in cell structure or adhesion.

In search of candidate nuclear matrix architectural transcription factors, we recently identified and cloned a family of nuclear matrix DNA-binding proteins, NP/NMP4 [Alvarez et al., 1997, 1998; Thunyakitpisal et al., in review]. These proteins bind to the rat type I collagen α1(I) polypeptide chain (*COL1A1*) promoter in the manner of architectural transcription factors, i.e. they bind within the minor groove of the poly (dT) consensus sequence and bend the promoter DNA [Alvarez et al., 1997, 1998; Thunyakitpisal et al., in review]. NP/NMP4 are in-frame splice variants of a single rat gene; they are expressed in embryonic nerve and bone, and exhibit a wide tissue distribution in the adult. Transcripts for all cloned splice variants of NP/NMP4 are expressed in rat bone and UMR-106 rat osteoblast-like cells [Thunyakitpisal et al., in review]. These Cys_2His_2 zinc finger proteins contain an AT-hook domain that facilitates changes in DNA structure [Aravind and Landsman, 1998]. The NP/NMP4 isoforms have from five to eight zinc fingers [Thunyakitpisal et al., in review]. Interestingly, isoform 28H does not exhibit any appreciable *COL1A1* binding [Thunyakitpisal et al., in review]. Mutation of the NP/NMP4 binding sites upregulates *COL1A1* promoter activity in UMR-106 cells [Thunyakitpisal et al., in review].

NP/NMP4 also associate with focal adhesions [Nakamoto et al., 2000] consistent with our hypothesis that these proteins transduce changes in cytoskeletal organization and adhesion into changes in promoter geometry. Some of the isoforms of NP/NMP4 are identical to those of the recently cloned protein CIZ (Cas-interacting zinc finger protein), a nucleocytoplasmic shuttling protein [Nakamoto et al., 2000]. CIZ was identified by far-Western analysis as a binding partner of the Cas-SH3 domain [Nakamoto et al., 2000]. Although Cas binds to focal adhesions and is essential for assembling actin filaments [Honda et al., 1998], CIZ was observed predominantly in the nucleus and regulated the activity of MMP promoters in NIH3T3 cells [Nakamoto et al., 2000].

In this study, we used antibodies to NP/NMP4 and green fluorescent protein (GFP)-NP/NMP4 fusion proteins to map the osteoblast cellular and subnuclear distribution of these isoforms and to identify their nuclear localization and nuclear matrix targeting signals. The present data suggest that the arrangement of the NP/NMP4 zinc fingers largely determines the subnuclear location of these isoforms in the osteoblast.

MATERIALS AND METHODS

Enhanced Green Fluorescent Protein (GFP)-NP/NMP4 Expression Vectors

Expression vectors for GFP-NP/NMP4 fusion proteins were prepared by polymerase chain reaction (PCR) amplification of full-length cDNAs and truncated derivatives, and then cloned into pEGFP-C1 (Clontech, Palo Alto, CA). All vector DNA was isolated from transformed DH5α competent cells (GibcoBRL, Grand Island, NY) using MiniPrep Express™ (Bio101, Inc., Vista, CA). *Pfu*-DNA polymerase (Stratagene, La Jolla, CA) was used for preparing GFP-NP/NMP4 fusion constructs. Oligonu-

cleotide primers from Sigma-Genosys, (The Woodlands, TX) were used to generate these constructs. The integrity of all plasmids was confirmed by DNA sequencing and restriction enzyme digestion.

Western analysis of 293T cell lysates containing some of the GFP-NP/NMP4 constructs confirmed the predicted molecular weights of the expressed fusion proteins. Whole cell protein extracts obtained from cells transfected with the GFP-NP/NMP4 fusion constructs were electrophoresed on 12% SDS-PAGE gels and the proteins transferred to a PVDF membrane (BIORAD, Hercules, CA) as described [Meier et al., 1999]. Membranes were blocked with 1% bovine serum albumin in TBS-T (room temperature, 1 h) and incubated with a Living Colors® Peptide antibody-HRP Conjugate (Clontech) at 1:400. SuperSignal® West Pico chemiluminescent substrate (Pierce, Rockford, IL) was used for GFP antigen detection.

Cell Culture and Transient Transfections

Osteoblast-like cells. UMR 106-01 rat osteosarcoma cells, (kindly donated by Dr. Nicola Partridge, St. Louis University, St. Louis, MO), were grown in MEM media supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin, 2 mM L-glutamine (Gibco-BRL), and 10% fetal bovine serum (FBS; Sigma). Cells were seeded at $4–5 \times 10^3$ cells/cm^2 in sterile single chamber slide flaskettes (Nalge Nunc Intl; Naperville, IL), 24 h prior to transfection. All cells were maintained in humidified 95% air/5% CO_2 at 37°C. Cells were transiently transfected with 1.5 μg GFP-NP/NMP4 plasmid DNA using LipofectAMINE PLUS™ (Gibco BRL) and at least 48 h after transfection, fixed either as whole cell or extracted preparations (see below), stained, and mounted for immunofluorescence microscopy.

Human embryonic kidney 293T cells (ATCC, Rockville, MD). Cells were maintained in DMEM (Gibco BRL) media supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Irvine Scientific). For each vector construct, cells were plated into three 60 mm culture dishes at an initial density of 1.5×10^5 cells/dish. Cells were transfected with 10 μg vector DNA/dish using the CalPhos system (Clontech). Control cultures received empty expression vector or were mock transfected. Whole cell lysates were harvested 48 h after transfection as previously described [Meier et al., 1999].

In Situ Sequential Extraction

The nuclear matrix was isolated in situ by sequential extraction of the soluble, cytoskeletal, and chromatin proteins [He et al., 1990; Zeng et al., 1994]. Briefly, cells were rinsed with PBS. Soluble cytosolic and membrane proteins were removed by exposure (3×2 min, 4°C) to CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 4 mM vanadyl ribonucleoside complex (Gibco BRL), 1.2 mM phenylmethylsulfonyl fluoride, 2.1 μM leupeptin, and 1.4 μM pepstatin A; Roche Molecular Biochemicals (Indianapolis, IN). Chromatin then was removed by incubating cells in CSK buffer containing 30 U RNAse free, DNAse I (40 min, 32–34°C; Roche Molecular Biochemicals) followed by incubation in CSK containing 0.25 M ammonium sulfate (5 min, 4°C). Cells then were extracted with high salt by incubating in 2 ml CSK buffer and gradually adding an equal volume of 4 M NaCl dropwise over a period of 1 h while shaking. Lastly, cells were placed on ice for 4 min, fixed in 3.7% formaldehyde/CSK for 15 min, and processed for immunodetection of NuMA and chromatin staining as described below. In some experiments, transfected cells were fixed after the initial CSK extraction or DNAse I digestion steps.

Immunocytochemistry and Immunofluorescence Light Microscopy

Preparations of whole (unextracted) cells were fixed in 3.7% formaldehyde (Ladd Research Industries, Inc., Burlington, VT)/PBS for 15 min at room temperature and permeabilized in PBS-T (PBS containing 0.1% Triton X-100, Roche Molecular Biochemicals) for 5 min. To assess the integrity of the nuclear matrix fixed cells (whole or extracted) were blocked in PBS containing 10% FBS for 20 min, and subsequently incubated in anti-human NuMA purified mouse monoclonal antibody [1:100 in P-BSA, Oncogene Science, Cambridge, MA] for 1 h at 37°C as previously described [Torrungruang et al., 1998]. NuMA antibody was detected with secondary anti-mouse immunoglobulin (IgG) conjugated to Texas Red (1:1,000 in P-BSA, 30 min at 37°C, Jackson

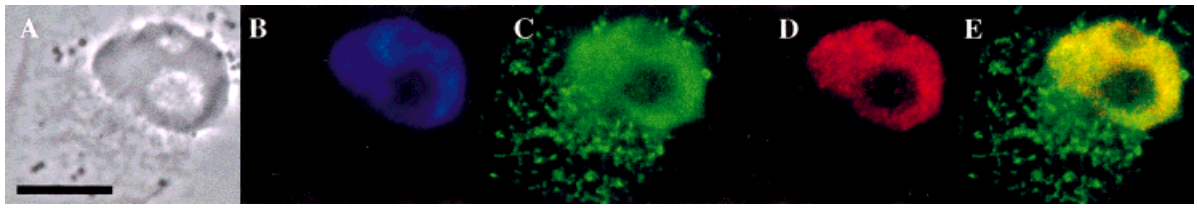


Fig. 1. Immunocytochemistry demonstrates that the NP/NMP4 proteins localize to both the cytosol and nucleus of UMR-106 cells. **A**: Phase contrast image of UMR-106 cell; **B**: DAPI staining of same cell showing nuclear DNA; **C**: Staining of same cell with polyclonal antibodies to the amino terminus of isoform 11H (green). Note a punctate pattern in the cytosol with heaviest concentration in the nucleus. Treatment of cells with pre-immune serum exhibited no staining (data not shown); **D**: Staining of same cell with antibodies to nuclear matrix protein NuMA (red); **E**: Pseudocolor overlay showing relative distributions and overlap (yellow) of NP/NMP4 proteins and NuMA. Scale bar = 10 μM.

ImmunoResearch Labs, West Grove, PA). To characterize native NP/NMP4 subcellular localization, cells were incubated with a polyclonal antibody raised against the amino terminus GST-fusion protein, derived from clone 11H (1:200 in P-BSA, 1 h 37°C) and subsequently labeled with secondary anti-rabbit IgG conjugated to FITC (1:1,000 in P-BSA, 30 min at 37°C). The presence or absence of nuclear DNA was assessed by exposing cells to 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 5 μg/ml in PBS containing 0.1% Triton X-100, Sigma) as described [Feister et al., 1997]. All cell preparations were mounted in aqueous mounting media (Quantafluor® Mounting Medium, Kallestad, Chaska, MN) and stored at 4°C prior to analysis with immunofluorescence microscopy.

Phase contrast and fluorescent digital images were captured using a Zeiss Axiovert TV light microscope (Thornwood, NY) in combination with a phase contrast 100× (NA1.3) oil-immersion lens. All fluorescent images of triple-labeled cells were acquired using narrow band-pass rhodamine, fluorescein, and DAPI filters and a charge-coupled device (CCD) camera (Photometrics, Inc., Tucson, AZ). IPLab Spectrum software (Version 3.1, Signal Analytics, Vienna, VA) was used to capture, process, and RGB color enhance images. Micrographs were montaged for presentation using Adobe Photoshop 4.0 (Adobe Systems, Inc., San Jose, CA).

RESULTS

NP/NMP4 Localizes to Both the Cytosol and Nucleus of Osteoblast-Like Cells

To determine the cellular distribution of the NP/NMP4 proteins, we first used a polyclonal antibody to the amino terminus of clone 11H to stain UMR-106 cells (Fig. 1). For each experiment, cells were viewed under phase contrast (Fig. 1A) and stained with DAPI to show nuclear DNA (Fig. 1B), and with a polyclonal antibody to detect the NP/NMP4 proteins (Fig. 1C), and with a monoclonal antibody to the nuclear matrix protein NuMA (Fig. 1D). The NP/NMP4 staining pattern observed in the osteoblast-like cells exhibited a punctate pattern in the cytosol and a heavy concentration in the nucleus (Fig. 1). This distribution is similar to that reported for CIZ expression in the normal rat fibroblast 3Y1 cell line, in which CIZ was detected in focal adhesions as well as the nucleus [Nakamoto et al., 2000]. A pseudocolor overlay demonstrated a spatial overlap between the nuclear matrix protein NuMA and the NP/NMP4 proteins within the nucleus (Fig. 1E). NuMA is a component of the spindle pole apparatus in dividing cells and comprises the nuclear substructure of some non-dividing cells, including osteoblasts [Feister et al., 2000; Torrungruang et al., 1998].

NP/NMP4 Splice Variants Exhibit Distinct Osteoblast Nuclear Distributions

In light of recent data indicating a correlation between nuclear factor function and localization [Pombo et al., 1998; Lelièvre et al., 1998; Lelièvre and Bissell, 1998; Larsson et al., 1995], we investigated the nuclear distributions of the NP/NMP4 isoforms. To map the osteoblast subnuclear localization of the NP/NMP4 splice variants, UMR-106 cells were transiently transfected with expression vectors for the full-length NP/NMP4 isoforms (11H, 13H, 21H, and 28H) fused to the enhanced green fluorescent protein (GFP). For each ex-

periment, transfected cells were co-stained with DAPI and the monoclonal antibody to the nuclear matrix protein NuMA. Cells transfected with GFP vector only (control), exhibited diffuse cyto- and nucleoplasmic staining. Immunofluorescence microscopy indicated that GFP-11H was localized exclusively within the nucleus (Fig. 2A,B). The fusion protein GFP-11H had a diffuse nuclear distribution and was absent from nucleoli. The absence of GFP-11H from the cytosol is consistent with previous observations that the predominant localization of these proteins is to the nucleus, particularly under conditions of overexpression [Nakamoto et al., 2000]. Pseudocolor overlay images of NuMA and GFP-11H indicate that both proteins are excluded from the nucleoli but that the overlap of NuMA and GFP-11H (yellow color, Fig. 2B) is not complete (green color). The GFP-13H fusion protein exhibited an identical nuclear distribution to GFP-11H (Fig. 2A,B). Isoform 13H is identical to 11H, in sequence, with the exception of a 16 amino acid deletion proximal to the amino terminus (Fig. 2A). About 90% of the cells transfected with the isoform GFP-21H fusion protein exhibited fluorescence primarily concentrated within two distinct, non-nucleolar foci, with minor amounts diffusely distributed within the non-nucleolar nucleus (Fig. 2A,B). The non-nucleolar foci are accentuated when viewed as the pseudocolor overlay with NuMA (Fig. 2B). The remaining 10% of the cells transfected with the GFP-21H construct exhibited the fluorescent pattern observed with isoforms 11H and 13H. Isoform 21H is identical to 11H except for the deletion of zinc fingers 4 and 5 (Fig. 2A). Like isoforms 11H and 13H, this isoform of NP/NMP4 exhibited sequence-specific binding to the *COL1A1* promoter [Thunyakitpisal et al., in review]. By contrast, the isoform GFP-28H protein was weakly expressed in the nucleoplasm and highly concentrated in the nucleoli (Fig. 2A,B). Approximately 10% of the cells transfected with GFP-28H exhibited a fluorescent pattern similar to that observed with 11H and 13H. GFP-28H targeting of the nucleoli was particularly evident in the pseudocolor overlay images demonstrating its contrasting subnuclear compartmentalization with NuMA (Fig. 2B), a protein that is excluded from the nucleolus in osteoblasts [Torrungruang et al., 1998]. Isoform 28H contains a 31 amino acid insertion prior to the first zinc finger and is truncated immediately downstream of the fifth zinc finger, lacking zinc fingers 6, 7, 8 and the QA-repeat domain (Fig. 2A). This isoform exhibited little binding to the *COL1A1* promoter [Thunyakitpisal et al., in review].

The Zinc Finger Domain Mediates Nuclear Localization of the NP/NMP4 Proteins

To determine the nuclear localization domain(s) of the NP/NMP4 proteins, we prepared GFP expression constructs containing either the full-length or truncated versions of isoform 11H (Fig. 3A). The predicted molecular weights of some of these isoforms were confirmed by Western analysis (Fig. 3B, and data not shown). These expression vectors were transiently transfected into UMR-106 cells and the expressed fusion proteins viewed in fixed, whole (unextracted) cells with phase and immunofluorescence microscopy (Fig. 3C). GFP-fusion proteins lacking the zinc finger domain exhibited diffuse distribution throughout the cyto- and nucleoplasm of the UMR-106 cells (Fig. 3C). The amino terminus region (amino acids 1–187, Fig. 3A,C) and the amino terminus + the AT-hook domain (amino acids 1–230, Fig. 3A,C) of the NP/NMP4 proteins were not sufficient to localize the GFP signal exclusively to the nucleus. The GFP-fusion protein that included these regions in addition to the zinc finger domain, but lacking the QA-repeat motif (GFP-11H[1–461]), exhibited exclusive nuclear localization indistinguishable from the full-length control (Fig. 3A,C). Small proteins ($\leq$60 kD) can passively diffuse into the nucleus and this may be the mechanism by which the fusion proteins GFP-11H[1–187] ($\approx$49.5 kD) and GFP-11H[1–230] ($\approx$55 kD) entered the nucleus. However, larger proteins require the presence of a nuclear localization signal for regulated nuclear import [Nakielny and Dreyfuss, 1997; LaCasse and Lefebvre, 1995; Schmidt-Zachmann and Nigg, 1993] and therefore GFP-11H[1–461] ($\approx$80 kD) likely required such a signal to concentrate within the nucleus. To determine whether the zinc finger domain alone could direct nuclear localization, UMR-106 cells were transfected with the construct that contained zinc fingers #1 through #8 (GFP-[1–8]ZF). This fusion protein localized exclusively within the cell nucleus similar to the distribution of the full length fusion protein (Fig. 3A,C). Although both the GFP-[1–8]ZF and GFP-11H[1–230] are 55 kD proteins and

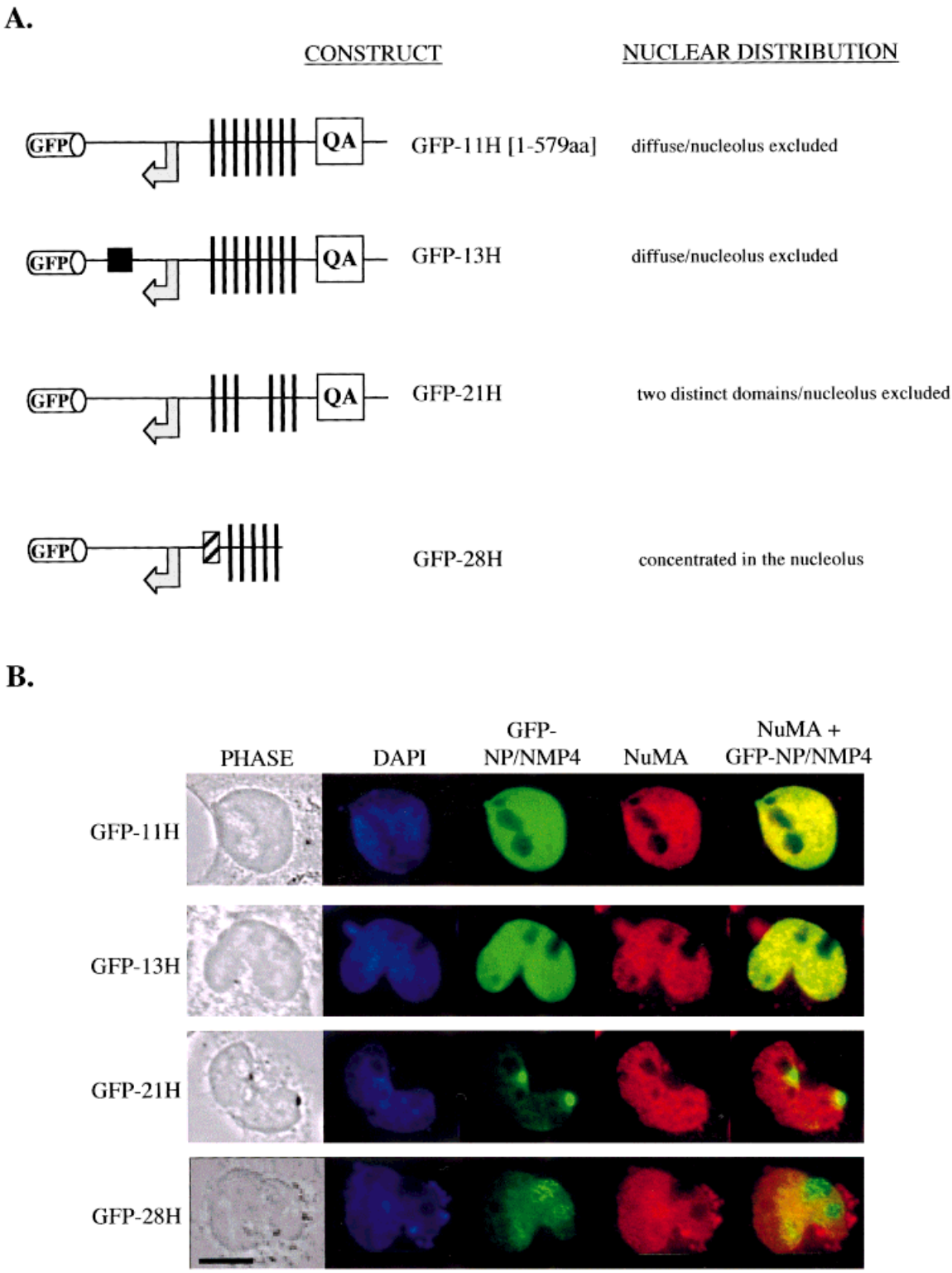


Fig. 2. NP/NMP4 splice variants exhibit distinct osteoblast nuclear distributions. **A**: Schematic representation and nuclear distributions of NP/NMP4 isoforms 11H, 13H, 21H, and 28H. GFP, green fluorescent protein; arrow, AT-hook domain; vertical dark lines, Cys_2His_2 zinc fingers; QA, glutamine/alanine repeat; black box,16-amino acid deletion in the amino terminal region of isoform 13H; checkered box, 30-amino acid insert in isoform 28H. **B**: Fixed (whole cell) UMR-106 cells transiently transfected with expression vectors containing GFP-NP/NMP4 fusion proteins. Cells transfected with the GFP-constructs (green) also were stained with DAPI (blue) to show nuclear DNA, and immunolabelled with antibody to the nuclear matrix protein NuMA (red); pseudocolor overlay of the same cells showed juxtaposition of both the GFP-NP/NMP4 isoform and NuMA (yellow). GFP-11H and 13H exhibited a diffuse nuclear distribution with exclusion from the nucleolus. GFP-21H concentrated within two distinct, non-nucleolar foci with minor amounts diffusely distributed within the non-nucleolar nucleus. GFP-28H primarily localized to the nucleolus, in contrast to NuMA, with some minor distribution of protein throughout the non-nucleolar nucleus. Scale bar = 10 μm.

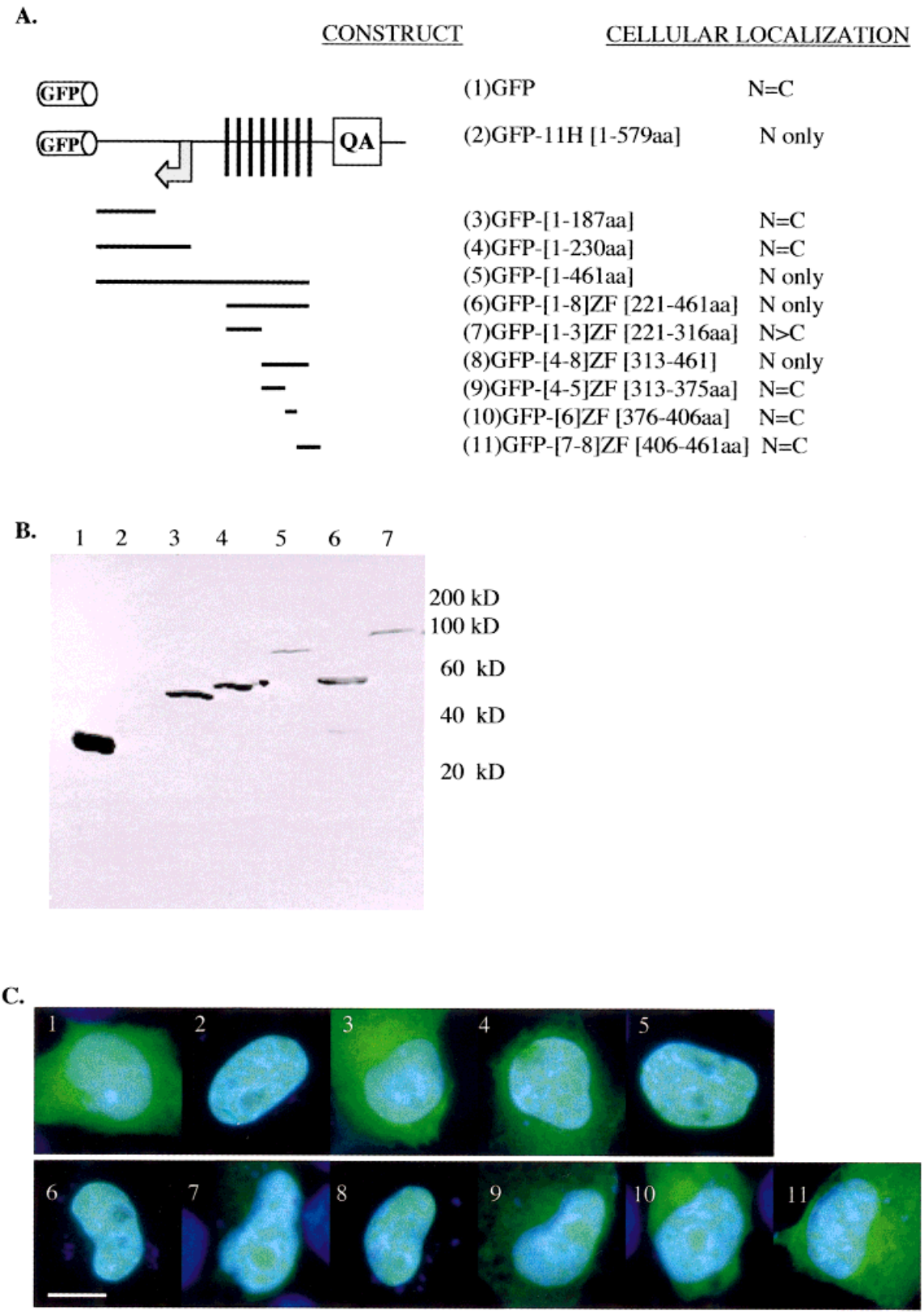

A.
CONSTRUCT
CELLULAR LOCALIZATION
GFP
GFP
QA
(1)GFP N=C
(2)GFP-11H [1-579aa] N only
(3)GFP-[1-187aa] N=C
(4)GFP-[1-230aa] N=C
(5)GFP-[1-461aa] N only
(6)GFP-[1-8]ZF [221-461aa] N only
(7)GFP-[1-3]ZF [221-316aa] N>C
(8)GFP-[4-8]ZF [313-461] N only
(9)GFP-[4-5]ZF [313-375aa] N=C
(10)GFP-[6]ZF [376-406aa] N=C
(11)GFP-[7-8]ZF [406-461aa] N=C
B.
1 2 3 4 5 6 7
200 kD
100 kD
60 kD
40 kD
20 kD
C.
1 2 3 4 5
6 7 8 9 10 11

Figure 3

are capable of passive nuclear entry, only GFP-[1–8]ZF localized exclusively to the nucleus. This observation confirmed that the multiple zinc finger domain was necessary and sufficient for nuclear localization.

To further delimit the nuclear localization signal, we made several constructs containing subsets of the eight zinc fingers (Fig. 3A,C). The only truncated derivative that demonstrated exclusive nuclear staining was the construct that contained zinc fingers #4 through #8 (GFP-[4–8]ZF), although the first three zinc fingers (GFP-[1–3]ZF) exhibited some nuclear targeting (Fig. 3A,C). Interestingly, the distinct nuclear and nucleolar distributions of the parent isoforms (11H/13H, 21H, and 28H) could not be duplicated with the truncated zinc finger constructs (Fig. 3A,C). To test whether the unique 31 amino acid insert of isoform 28H was responsible for mediating the nucleolar targeting of this protein, we prepared a GFP construct containing this fragment. This construct exhibited diffuse nuclear and cytoplasmic staining and thus was demonstrated not to act as a nucleolar targeting signal (data not shown).

The Zinc Finger Domain Mediates Nuclear Matrix Targeting of the NP/NMP4 Proteins But DNA Binding is Not Required

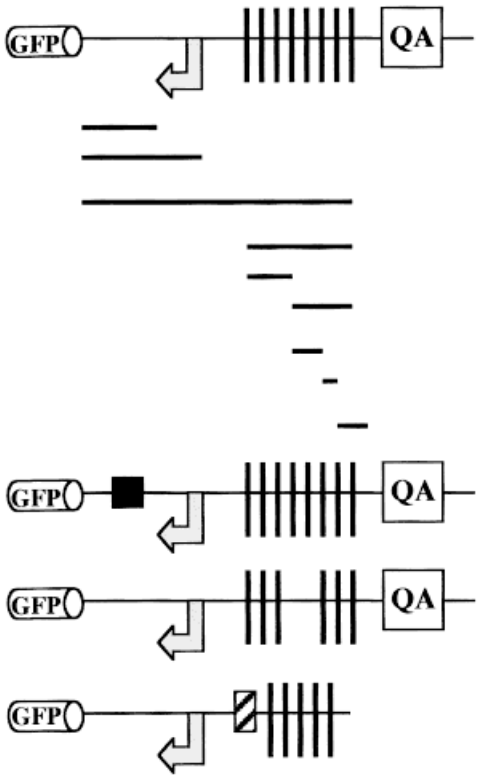
To determine the domain(s) responsible for targeting the NP/NMP4 proteins to the nuclear matrix, we sequentially removed soluble cytoplasmic proteins, DNA, and chromatin proteins from UMR-106 cells transiently transfected with the GFP-NP/NMP4 fusion constructs described above. The remaining salt-resistant core nuclear matrix consists of insoluble structural and regulatory proteins as well as hnRNA [He et al., 1990]. The integrity of this structure was confirmed by immunodetection of the structural filament nuclear matrix protein, NuMA [Zeng et al., 1994]. The presence or absence of DNA was determined by staining the cells with DAPI. The GFP-11H constructs containing the amino terminal region (amino acids 1–187) and the amino terminal + the AT-hook domain (amino acids 1–230) were extracted during removal of the soluble cell fraction with CSK buffer (Fig. 4A), as was the GFP protein alone (data not shown). NuMA and nuclear DNA were not removed with this step in the extraction protocol. The full-length GFP-11H protein was retained within the nuclear matrix after DNAse I digestion and high salt extraction and appeared diffusely distributed within the core matrix fraction, except for the nucleolar remnant, (Fig. 4A,B). The retention of the GFP-11H[1–461], GFP-[1–8]ZF, and GFP-[4–8]ZF fusion proteins in the nuclear matrix after DNAse I digestion and high salt extraction (Fig. 4A) indicated that the zinc finger domain was important for nuclear matrix targeting. Additionally, these experiments demonstrated that zinc fingers #4 through #8 were sufficient for retention by the nuclear matrix and that nuclear matrix retention of isoform 11H did not depend on DNA-binding, since DAPI staining indicated an absence of nuclear DNA (Fig. 4B). The other NP/NMP4 isoforms (13H, 21H, and 28H) and the native NP/NMP4 proteins as indicated by antibody staining, also were retained in the nuclear matrix fraction of UMR-106 cells (Fig. 4A).

Fig. 3. The zinc finger domain of isoform 11H is necessary and sufficient to target the GFP-11H fusion protein to the osteoblast nucleus. **A**: Schematic representation of isoform GFP-11H, its truncated derivatives, and their cellular localization in UMR-106 cells. **B**: Western analysis confirms predicted size of GFP-NP/NMP4 fusion constructs. Lane 1: GFP only, predicated molecular weight, 29kD; Lane 2: cells only; Lane 3: GFP-11H[1–187], predicted molecular weight, 49.5kD; Lane 4: GFP-11H[1–230], predicted molecular weight, 55kD; Lane 5: GFP-11H[1–461], predicted molecular weight, 80kD; lane 6: GFP-[1–8]ZF [221–461], predicted molecular weight, 55kD; Lane 7: GFP-11H (full-length isoform), predicted molecular weight, 92kD. The numbers in brackets refer to the amino acid fragment included in the fusion protein. These fusion proteins were expressed in transfected 293T cells (see Materials and Methods for details). **C**: Pseudocolor overlays of DAPI and GFP-11H constructs in transiently transfected UMR-106 osteoblast-like cells. The numbers of the panels refer to the number of the construct listed in 3A. The only constructs to exhibit exclusive nuclear staining were the GFP-11H full-length construct [1–579aa], GFP-11H[1–461aa], which lacks the QA-repeat domain but contains the eight zinc fingers, GFP-[1–8]ZF[221–461], containing only the eight zinc fingers, and GFP-[4–8]ZF[313–461aa], containing zinc fingers #4–8. Scale bar = 10 μm.

DISCUSSION

The four NP/NMP4 isoforms have distinct osteoblast nuclear distributions largely mediated by the organization of their zinc fingers. The lack of cytoplasmic localization of the GFP-fusion constructs is consistent with the behavior of these proteins under conditions of overexpression [Nakamoto et al., 2000]. The diffuse distribution of isoforms 11H and 13H is similar, but not identical, to that of NuMA, a nuclear matrix protein that forms a scaffold in interphase osteoblasts

A.



CONSTRUCT	NM ASSOCIATION
GFP-11H [1-579aa]	YES
GFP-[1-187aa]	NO
GFP-[1-230aa]	NO
GFP-[1-461aa]	YES
GFP-[1-8]ZF [221-461aa]	YES
GFP-[1-3]ZF [221-316aa]	NO
GFP-[4-8]ZF [313-461]	YES
GFP-[4-5]ZF [313-375aa]	NO
GFP-[6]ZF [376-406aa]	NO
GFP-[7-8]ZF [406-461aa]	NO
GFP-13H	YES
GFP-21H	YES
GFP-28H	YES
NATIVE PROTEINS	YES

B.

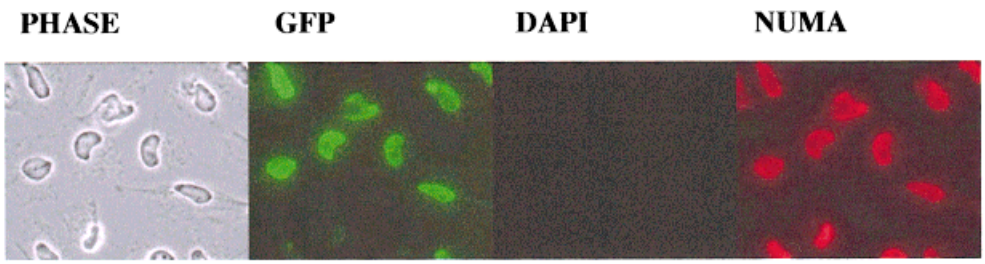


Fig. 4. The zinc finger domain of isoform 11H is necessary and sufficient to target the GFP-11H fusion protein to the osteoblast nuclear matrix. **A**: Schematic representation of isoform GFP-11H, its truncated derivatives, isoforms 13H, 21H, and 28H, and degree of association with the UMR-106 nuclear matrix following in situ sequential extraction. The only constructs to exhibit association with the nuclear matrix were the GFP-11H full-length construct [1–579aa], GFP-11H[1–461aa], which lacks the QA-repeat domain but contains the eight zinc fingers, GFP-[1–8]ZF[221–461], containing only the eight zinc fingers, and GFP-[4–8]ZF[313–461aa], containing zinc fingers #4–8. The other isoforms GFP-13H, GFP-21H, and GFP-28H, as well as the native proteins, also remained associated with the nuclear matrix after sequential extraction. **B**: Cells transfected with the GFP-constructs (green) were stained with DAPI (blue) to show nuclear DNA, and immunolabelled with antibody to the nuclear matrix protein NuMA (red), after being subjected to in situ sequential extraction. The full-length isoform GFP-11H [1–579aa] remains within the nucleus after DNase I digestion and high salt extraction. Note the absence of DNA (no DAPI staining), but the GFP-11H and NuMA remain associated with the nuclear matrix. Scale bar = 10 μm.

[Feister et al., 2000; Torrungruang et al., 1998]. Although fluorescent images indicate that NuMA and isoforms 11H and 13H are diffusely distributed throughout the nucleus and excluded from the nucleolus, pseudocolor overlay images reveal that there are regions of non-overlap between these proteins. The 11H and 13H isoforms have an identical motif of eight Cys_2His_2 zinc fingers. Also, the 16-amino acid deletion in the amino terminal region of isoform 13H does not alter its ability to target the nucleus or modify its nuclear compartmentalization as compared to 11H. Isoform 21H has six zinc fingers, #4 and #5 are absent, and is otherwise identical to isoform 11H. Nevertheless, this protein largely localizes within two distinct nuclear foci. This pattern is similar to that described for the OPT domain (Oct1/PTF/transcription domain) characterized in HeLa cells [Pombo et al., 1998]. The transcription factors PTF and Oct1 concentrate in one or very few discrete regions colocalizing with a small region on chromosome 6 (band 6p21). It has been proposed that the OPT domain brings together snRNA genes to a region where the appropriate transcription and processing factors are concentrated, similar to the localization of ribosomal genes in the nucleolus [Pombo et al., 1998]. Finally, isoform 28H has only the first five zinc fingers of clones 11H and 13H, and this isoform localizes to the nucleolar region of the UMR-106 cells. Similarly, the ubiquitous transcription factor YY-1 has four Cys_2His_2 zinc fingers and localized within the nucleolus of HeLa S_3 and SaOS-2 osteoblast-like cells [McNeil et al., 1998; Guo et al., 1995]. Although the organization of the zinc fingers plays the most significant role in mediating the subnuclear localization of the NP/NMP4 isoforms, other regions of these proteins clearly contribute, since the distinct nuclear and nucleolar distributions of the parent clones (11H/13H, 21H, and 28H) could not be fully duplicated with the truncated zinc finger constructs.

In similar fashion to our observations of NP/NMP4 isoforms, alternative splicing within the Cys_2His_2 zinc finger domain mediates distinct subnuclear localization of isoforms of the WT1 tumor suppressor protein [Larsson et al., 1995]. Two alternative splice sites in the WT1 transcript permit the encoding of four proteins that each carry four zinc fingers [Larsson et al., 1995]. Lysine, threonine, and serine (KTS) may be included or excluded between the third and fourth zinc fingers and it is the presence or absence of these amino acids within the context of the zinc fingers that targets the protein to a particular nuclear subdomain [Larsson et al., 1995]. In the mesonephric cell line, M15, WT1 (+KTS) exhibited a "speckled" nuclear profile and associated with snRNPs, suggesting a role in mRNA processing [Larsson et al., 1995]. WT1 (-KTS) localized to distinct nuclear foci with other transcription factors including Sp1 [Larsson et al., 1995]. Additionally, these proteins associate with the nuclear matrix [Dobashi et al., 1997]. The WT1 isoforms appear to have distinct functional roles in transcription and splicing which are mediated, in part, by their targeting of distinct nuclear subcompartments. A correlation between the subnuclear compartmentalization of NP/NMP4 isoforms and their function, with respect to *COL1A1* transcription, is not yet apparent. Further, the fact that the NP/NMP4/CIZ proteins likely regulate multiple genes [Nakamoto et al., 2000; Thunyakitpisal et al., in review] may prevent a simplistic interpretation of the spatial data.

Our data indicate that a minimum of five zinc fingers (#1–5 or #4–8) are required for targeting the NP/NMP4 proteins to the osteoblast nucleus and nuclear matrix. Any GFP-construct lacking the zinc finger motif or containing any single zinc finger, or combination of less than five zinc fingers, did not localize exclusively to the nucleus and were not retained in the residual nuclear matrix upon extraction. Additionally, the 31 amino acid insertion prior to the first zinc finger in isoform 28H did not mediate nucleolar localization. Recent studies have determined that the Cys_2His_2 zinc finger domain mediates nuclear localization in numerous transcription factors including WT-1 [Bruening et al., 1996], Egr-1 [Gashler et al., 1993], OZF [Ferbus et al., 1996], and JAZ [Yang et al., 1999]. This domain also contributes to nuclear localization of the trans-acting proteins GATA-4 [Morrisey et al., 1997] and the gut-enriched Krüppel-like factor, GKLF [Shields and Yang, 1997]. Finally, as was determined for NP/NMP4 proteins in this study, the zinc finger motif mediated both nuclear localization and nuclear matrix targeting in the transcription factors YY-1 [Austen et al., 1997; Bushmeyer and Atchison, 1998; McNeil et al., 1998] and ZNF74 [Grondin et al., 1996, 1997].

Nuclear matrix targeting of NP/NMP4 by the zinc finger domain does not require DNA-

binding indicating these proteins associate directly with the proteinaceous scaffold or the residual hnRNA of the nucleoskeleton. Treatment of the osteoblast-like cells with DNase I and extraction with ammonium sulfate removed the nuclear DNA, as confirmed by DAPI staining, but did not dislodge the GFP-NP/NMP4 fusion proteins or the native NP/NMP4 proteins, as determined by antibody staining. A similar DNA-independent association with the nuclear matrix via a portion of the zinc finger domain was observed with YY-1 [McNeil et al., 1998]. RBF-1 (receptor binding factor 1), is a 10 kD nuclear matrix protein that acts as an acceptor site for the avian oviduct progesterone (Pg) receptor [Spelsberg et al., 1996]. This acceptor protein binds to an AT-rich domain flanked by GC-rich sequences in the promoter region of the c-myc proto-oncogene and has been proposed to mediate the Pg-induced down regulation of c-myc gene expression [Spelsberg et al., 1996]. Similarly, there may be nuclear matrix acceptor proteins that recognize certain Cys_2His_2 zinc finger domains or alternatively, proteins like NP/NMP4 may themselves be nuclear matrix acceptor proteins that mediate the assembly of higher order nucleoprotein complexes along specific promoters.

The localization of NP/NMP4/CIZ within the cytoplasm, focal adhesions [Nakamoto et al., 2000], and in distinct nuclear matrix subcompartments may be part of the "solid state" signaling mechanism that relies on the structural continuity from the cell membrane to the target genes within the nucleus [Lelièvre et al., 1996; Lelièvre and Bissell, 1998]. The native NP/NMP4/CIZ proteins are predominantly located in the nucleus [this study, and Nakamoto et al., 2000]. The functional relationship between cytosolic and nuclear NP/NMP4, if any, remains to be determined. How these proteins compartmentalize between the cytosol and the nucleus is not yet clear. However, protein shuttling can occur via the slow diffusion out of the nucleus without any specific nuclear export sequence [Schmidt-Zachmann et al., 1993; Lelièvre and Bissell, 1998] and this may be the mechanism by which NP/NMP4/CIZ localizes to the cytoplasm. The distinct nuclear subdomains of the NP/NMP4 isoforms may comprise different transcriptional endpoints to mechanotransduction pathways. Alternatively, they may be different functional domains encompassing storage, transcription, or splicing. The study of NP/NMP4 may elucidate the nuclear events that transduce changes in tissue matrix organization into changes in gene expression involved in extracellular matrix remodeling.

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