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Lisse, Thomas S; Vadivel, Kanagasabai; Bajaj, S Paul; Zhou, Rui; Chun, Rene F; Hewison, Martin; Adams, John S

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The heterodimeric structure of heterogeneous nuclear ribonucleoprotein C1/C2 dictates 1,25-dihydroxyvitamin D-directed transcriptional events in osteoblasts

Thomas S Lisse, Kanagasabai Vadivel, S Paul Bajaj, Rui Zhou, Rene F Chun, Martin Hewison and John S Adams

Heterogeneous nuclear ribonucleoprotein (hnRNP) C plays a key role in RNA processing but also exerts a dominant negative effect on responses to 1,25-dihydroxyvitamin D (1,25(OH)2D) by functioning as a vitamin D response element-binding protein (VDRE-BP). hnRNPC acts a tetramer of hnRNPC1 (huC1) and hnRNPC2 (huC2), and organization of these subunits is critical to in vivo nucleic acid-binding. Overexpression of either huC1 or huC2 in human osteoblasts is sufficient to confer VDRE-BP suppression of 1,25(OH)2D-mediated transcription. However, huC1 or huC2 alone did not suppress 1,25(OH)2D-induced transcription in mouse osteoblastic cells. By contrast, overexpression of huC1 and huC2 in combination or transfection with a bone-specific polycistronic vector using a “self-cleaving” 2A peptide to co-express huC1/C2 suppressed 1,25D-mediated induction of osteoblast target gene expression. Structural diversity of hnRNPC between human/NWPs and mouse/rat/rabbit/dog was investigated by analysis of sequence variations within the hnRNP CLZ domain. The predicted loss of distal helical function in hnRNPC from lower species provides an explanation for the altered interaction between huC1/C2 and their mouse counterparts. These data provide new evidence of a role for hnRNPC1/C2 in 1,25(OH)2D-driven gene expression, and further suggest that species-specific tetramerization is a crucial determinant of its actions as a regulator of VDR-directed transactivation.

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hnRNPC1/C2-VDRE occupancy being displaced by the 1,25(OH)\textsubscript{2}D-bound VDR–RXR complex.\textsuperscript{8} In this capacity hnRNPC1/C2 functions as a dominant-negative-acting VDRE-binding protein (VDRE-BP). This action of hnRNPC1/C2 appears to contribute to normal VDR signaling capacity by occupying VDREs in the basal state and by participating in reciprocal cyclical VDRE binding following exposure to the VDR-activating ligand, 1,25(OH)\textsubscript{2}D. This reciprocal relationship between VDR and hnRNPC1/C2 is disrupted when the VDRE-BP is overexpressed, leading to 1,25(OH)\textsubscript{2}D insensitivity in the target cell.\textsuperscript{8,20–23} In vivo, over-expression of hnRNPC1/C2 has been associated with vitamin D-resistant rachitic bone disease in vitamin D-deprived New World primates\textsuperscript{24} and in man.\textsuperscript{7} Based on these molecular and phenotypic observations, the aim of the present study was to recapitulate and translate the VDRE-BP-induced vitamin D-resistant state in human bone cells by developing and characterizing murine osteoblastic cell lines which stably over express tetrameric HNRNPC proteins.

**MATERIALS AND METHODS**

Reagents and cell culture

Crystalline 1,25(OH)\textsubscript{2}D (Biomol, Plymouth Meeting, PA, USA) was reconstituted in absolute ethanol. Cells were treated with 0–10 nmol L\textsuperscript{-1} of 1,25(OH)\textsubscript{2}D incubated in the respective media with 1% FCS. MC3T3-E1 murine and MG63 human osteoblastic cells (both ATCC, Manassas, VA, USA) were maintained in α-MEM plus 10% FBS and incubated at 37°C in a 95% air/5% CO\textsubscript{2} atmosphere until 80%-90% confluent and then passaged. The J774A.1 mouse monocyte/macrophage cell line (ATCC) was cultured in DMEM with 10% FCS. For over-expression studies, the hnRNPC1 and C2 constructs were previously cloned and ligated into the pCDNA 3.1/V5-His-TOPO vector (Invitrogen, Grand Island, NY, USA); the previously cloned and ligated into the pCDNA 3.1/V5-His-TOPO vector (Invitrogen, Grand Island, NY, USA); the constructs were transfected using the BioT reagent (Bioland Scientific, Paramount, CA, USA) in 12-well tissue culture plates (0.1–1.0 µg per well total recDNA). Cells were analyzed at designated time points post transfection or transduction.

Cell fractionation and Western blot

Treated cells were harvested and washed with ice-cold phosphate-buffered saline. Cell lysates were prepared in RIPA buffer with 1 X ProteoBlock protease inhibitor cocktail (Fermentas, Glen Burnie, MD, USA) and 1 mmol L\textsuperscript{-1} PMSF. For separation of nuclear and cytoplasmic fractions for extraction, the NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) was utilized. Protein samples (20 µg) were separated by SDS-PAGE. Primary anti-hnRNCP C1/C2 (sc-32308; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and secondary antibodies were used at 1:1 000. 1-Block\textsuperscript{®} blocking reagent (Applied Biosystems, Foster City, CA, USA) was used during incubation steps.

Real-time quantitative PCR analysis

RNA was prepared using the RNeasy minikit (Qiagen Inc., Valencia, CA, USA). cDNA was synthesized by SuperScript Reverse Transcriptase III (Invitrogen) utilizing random hexamers. Quantitative PCR analysis was performed with a Stratagene MX-3005P instrument utilizing TaqMan\textsuperscript{®} system reagents from Applied Biosystems (Supplementary Table S1). Target genes were normalized to 18S rRNA expression. All cDNAs were amplified under the following conditions: 50 °C for 2 min; 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in triplicate.

Bicistronic human hnRNPC1 and C2 vector construction

Construct pRRL-sin-cPPT-mouse (mu) col1a1 2.3 kb promoter-IRES-GFP-hPGK-puromycin was produced by PCR of mu pcoll1a1 with XbaI at the 5’ end (primer 5-coll1a1-XbaI [Supplementary Table S2]) and blunt-ended at the 3’ end (primer 3-coll1a1-MluI) prior to digestion. The mouse collagen 2.3 kb promoter was kindly provided by Benoit de Crombrugge (Univ. Texas, M. D. Anderson Cancer Center). Construct pRRL-sin-cPPT-mu col1a1 2.3 kb promoter-human (hu) hnRNPC1-IRES-GFP-hPGK-puromycin was produced by PCR of hu hnrNPC1\textsuperscript{8} with a Mul site at the 5’ end (primer 5-hu hnrNPC1-Mul) and EcoRI at the 3’ end (primer 3-hu hnrNPC1-EcoRI). The products were then digested and ligated into pRRL-sin-cPPT-mu col1a1 2.3 kb promoter-IRES-GFP-hPGK-puromycin cut with Mul and EcoRI. Construct pRRL-sin-cPPT-mu col1a1 2.3 kb promoter-hu hnRNPC1-P2A-hu hnrNPC2-IRES-GFP-PGK-puromycin was produced by PCR of hu hnrNPC2 blunt-ended at the 5’ end (primer 5-hu hnrNPC2) and with Clal at the 3’ end (primer 3-hu hnrNPC2-Clal). This product was then digested and ligated with pRRL-sin-cPPT-CMV-GFP-P2A-MCS cut with SmaI and EcoRI. This plasmid was then used to produce P2A-hu hnrNPC2 blunt-ended at the 5’ end (primer 5-P2A) and with EcoRI at the 3’ end (primer 3-hu hnrNPC2- EcoRI). PCR of hu hnrNPC1 was produced with EcoRI at the 5’ end (primer 5-hu hnrNPC1-EcoRI) and blunt-ended without the stop codon at the 3’ end (primer 3-hu hnrNPC1-TAA). Both were digested and ligated into plasmid pRRL-sin-cPPT-mu col1a1 2.3 kb promoter-IRES-GFP-hPGK-puromycin digested with EcoRI. Corresponding empty vectors were also constructed.

Lentiviral production and transduction

Lentiviral production was performed for construct pRRL-sin-cPPT-mu col1a1 2.3 kb promoter-hnRNPC1-IRES-GFP-hPGK-puromycin. The construct pMD.G was used for the production of the VSV-G viral envelope in combination...
with the packaging constructs pHIt60 and the pBabe constructs correspond to the different transfer vectors. Briefly, 100 mm dishes of nonconfluent 293T cells were cotransfected with 6.5 μg of pHIt60, 3.5 μg of pMDG (encoding the VSV-G envelope) and 10 μg of p Babe (hu hnRNPC1 construct), by the CaPi-DNA coprecipitation method. Next day, the medium was adjusted to make a final concentration of 10 mmol-L\(^{-1}\) sodium butyrate and the cells were incubated for 8 h to obtain high-titer virus production as previously described. After the 8-h incubation, cells were washed and incubated in fresh medium without sodium butyrate. Conditioned medium was harvested 16 h later and passed through 0.45 mm filters. For transduction of MC3T3-E1 cells, cells were seeded at 8 × 10⁴ cells per well in 12-well plates. Transductions were carried out in 0.5 mL DMEM, including serial dilutions of lentiviral vector supernatant. The cells were then washed with phosphate-buffered saline after 24 h post transduction and incubated in regular medium for 48 h.

Molecular dynamic simulations and free energy calculations for hnRNPC
The coordinates of the human hnRNPC C leucine zipper-like oligomerization domain (CLZ) tetramer (PDB code 1TXP) were used for the simulations. PDB structure contains Ile at position 180 to get the correct human or mouse sequence, the Ile was mutated to Leu using the program PyMol (http://www.pymol.org). The mouse hnRNPC tetramer was built by mutating the residue Asn200 to Ser. Molecular dynamics simulations were performed in explicit water using NAMD with CHARMM27/CMAP force field. The tetramers were solvated in a cubic TIP3P solvent box of 9.0 Å. Periodic boundary conditions, Particle Mesh Ewald summation and SHAKE-enabled 2-femto seconds time steps were used in all molecular dynamics simulations. Initial configurations were subjected to a 500-step minimization with the harmonic constraints of 10 kcal mol\(^{-1}\)Å\(^{-2}\) on the protein heavy atoms. The systems were gradually heated from 0 to 300 K over a period of 30 ps with harmonic constraints. The simulation at 300 K was then continued for 200 ps during which the harmonic constraints were gradually lifted. The systems were then equilibrated for a period of 300 ps before the 10 ns production run. All simulations were carried out in the NPT ensemble. The binding free energy of the hnRNPC2 to hnRNPC1 for the different (inter-species) combinations was estimated using the MMPBSA module in AMBER 9.0 (Ref. 30) by taking snapshots (2000) at every 5 ps from the 10-ns production run.

Data normalization and statistical analysis
The real-time quantitative PCR methodology is based on determining the delta cycle threshold (ΔC_\text{T}) values. The average ΔC_\text{T} value was taken for triplicate sets. The fold induction was calculated by the formula: \(2^{(\text{avg}_{\text{C(T0nm)}} - \text{avg}_{\text{C(i)}})}\), where \(i\) is 0–10 nmol-L\(^{-1}\). An analysis of variance statistical test was conducted with a Bonferroni multiple comparison post hoc analysis where \(P≤0.05\) was considered statistically significant.

RESULTS
Effects of human hnRNPC1/C2 on 1,25(OH)\(_2\)D-mediated gene transactivation in mouse osteoblasts
As shown in Figure 1a, when transfected into mouse MC3T3 osteoblasts, suppression of 1,25(OH)\(_2\)D-mediated transcription was only observed when huC1 and huC2 were used in combination. In fact, expression of Ddit4, a gene we have previously shown to be induced by 1,25(OH)\(_2\)D in osteoblasts, was enhanced not suppressed, in mouse MC3T3 cells transfected with either huC1 or huC2 alone in the presence of 1,25(OH)\(_2\)D. By contrast, overexpression of either huC1 or huC2 was sufficient to suppress 1,25(OH)\(_2\)D-induced gene expression in human MG-63 osteoblast-like cells, with combined over-expression of huC1 and huC2 having an additive suppressive effect (Figure 1b).

To determine whether the lack of efficacy of huC1 and huC2 individually to squelch 1,25(OH)\(_2\)D-directed gene expression was due to relatively low transfection efficiency and subsequent expression of the human subunits in mouse osteoblasts, similar studies were also carried out using a bone-specific vector to drive expression of huC1 in mouse cells harboring the endogenously expressed oligomerization partner, muC2 (Figure 2a and 2b). Despite 50- to 3 000-fold higher levels of expression relative to the endogenous muC1 and muC2 expression levels, transfection with huC1 continued to have no significant effect on 1,25(OH)\(_2\)D-induced gene expression (Figure 2c).

The apparent requirement for both huC1 and huC2 to suppress 1,25(OH)\(_2\)D-directed transcription in mouse osteoblasts was further illustrated in MC3T3-transfected cell lines where huC1 and huC2 were co-expressed under the control of the osteoblast-specific mouse col1a1 promoter. In contrast to the previous experiments, vectors used in this part of the study encoded the huC1 and huC2 linked to one another by the self-cleaving 23 amino-acid picornaviral 2A-like sequence from the porcine teschovirus-1 (P2A) to allow for efficient functional stoichiometric expression of multiple flanking proteins under the influence of the bone-specific col1a1 2.3 kB promoter (Figure 3a). The advantage of combined transgene expression with the P2A over transgenes linked by an internal ribosomal entry site or introduced by individual constructs is its independence of copy number required for integration, its relatively small size and its high cleavage efficiency. Using this system, both the huC1 and huC2 isoforms were equally overexpressed specifically in the nuclear compartment of MC3T3 cells (Figure 3b). The
efficiency of transfection of the single open reading frame was assessed by green fluorescent protein (GFP) expression within various cell lines (Figure 4a), and the absence of uncleaved product in Western blot analyses indicated efficient cleavage of the P2A protein (Figure 4b). Cell (tissue)-specific expression of huC1/huC2 was confirmed by parallel analysis of the temporal pattern of endogenous mouse Col1a1 mRNA levels over time in MC3T3 bone cells, but not J774A mouse monocytes (24–96 h) (Figure 5).

As shown in Figure 3c, co-expression of huC1 and huC2 using the P2A vector acted as a dominant-negative inhibitor of 1,25(OH)₂D-induced expression of Cyp24a1 and Ddit4 in mouse MC3T3 osteoblastic cells and human MG-63 osteoblastic cells. Cells were treated with or without 10 nmol·L⁻¹ 1,25(OH)₂D for 6 h, 24 h post transfection with either empty vector (c), C1, C2 or C1 and C2 (C1/C2). Data show mean±s.d. (n=3 separate cultures) fold-change in mRNA expression for CYP24A1/Cyp24a1 and DDIT4/Ddit4 for each transfected cell type following treatment with 1,25(OH)₂D relative to vehicle-treated control cells. ***P<0.001, statistically different from empty vector controls (c), **P<0.01, statistically different from C.

Figure 1. Effects of overexpressed human hnRNPC1 and C2 on VDR-mediated gene transcription in human and mouse osteoblast-like cells. Effect of overexpression of human hnRNPC1 (C1) and/ or human hnRNPC2 (C2) on 1,25(OH)₂D-induction of the vitamin D-target genes CYP24A1/Cyp24a1 and DDIT4/Ddit4 in (a) mouse MC3T3 osteoblastic cells and (b) human MG-63 osteoblastic cells. Cells were treated with or without 10 nmol·L⁻¹ 1,25(OH)₂D for 6 h, 24 h post transfection with either empty vector (c), C1, C2 or C1 and C2 (C1/C2). Data show mean±s.d. (n=3 separate cultures) fold-change in mRNA expression for CYP24A1/Cyp24a1 and DDIT4/Ddit4 for each transfected cell type following treatment with 1,25(OH)₂D relative to vehicle-treated control cells. ***P<0.001, statistically different from empty vector controls (c), **P<0.01, statistically different from C.

Species-specific variations in the structure of the hnRNPC oligomerization domains dictate function

The hnRNPC proteins associate with RNA as tetramers with a composition of three hnRNP C1 subunits and one hnRNP C2 subunit (C₁₃–C₂₁). Studies in vivo have shown that the formation of the tetrameric complex, which is highly stable in solution, is driven by a 28-residue helical region (residues 180–207) referred to as hnRNPC leucine zipper-like (CLZ) oligomerization domain. The CLZ domain promotes the oligomerization by forming a coiled coil tetramer structure; hnRNPC proteins possessing mutations in the CLZ domain bind weakly to nucleic acid substrates. Amino-acid sequences of the hnRNPC CLZ oligomerization domain are well conserved within vertebrates, exhibiting 74%–98% identity to the human CLZ domain. Like many oligomerizing proteins, hnRNPC isoforms employ α-helical coiled coils as an oligomerization mechanism with the parts stabilized by continuous interhelical contacts formed between hydrophobic faces of amphipathic helices. For each set of seven residues (heptad) along the helix (denoted a–g; Figure 7b), the contact geometry is repeated for the ideal coiled coil. Hydrophobic residues occupying the a and d heptad positions form interhelical contacts in the coiled coil core as well as other contact surfaces at interfacial e and g heptad positions. Comparison of the human and mouse hnRNPC sequences in Figure 7b revealed that the Ser at position 200 in the mouse is replaced by Asp in man; as
such, the most distal interfacial g position heptad of the hnRNPC CLZ domain used for hnRNPC1–hnRNPC2 oligomerization in the mouse is disrupted.

Free energy and stability analysis of tetramers formed by combinations of mouse and human hnRNPC1/C2

To determine whether interaction between the Asp-200 in the human and Ser-200 in the mouse hnRNPC protein was sufficient to cause destabilization of the CLZ-coiled hnRNPC1/C2 tetramer, the binding free energy of interspecies tetramers harboring three hnRNPC1 and one hnRNPC2 subunit combinations, huC13–muC21 and muC13–huC21, was calculated using the MMPBSA method implemented in Amber 9.0. When calculating the binding free energy, the holotetramer was considered as a complex, the three hnRNPC1 subunits as a receptor and the

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Figure 2. Stable expression of only human hnRNPC1 protein in mouse MC3T3-E1 osteoblasts does not confer resistance to 1,25(OH)2D. (a) Osteoblastic transduction was performed using the lentivirus RRL transfer vector harboring a stop codon-less human (hu) hnRNPC1 cDNA and a GFP cassette driven by the bone-specific mouse col1a1 2.3 kB promoter. The lentivector backbone also contains the cPPT from the HIV-1 integrase gene to increase the copy number of lentivirus integrating into the host genome to enhance viral titer. (b) FACS sorting of GFP-positive MC3T3-E1 osteoblasts transduced with the hu hnRNPC1 and empty lentivirus vectors for the isolation and generation of stable clones. (c) Fifteen hu hnRNPC1-overexpressing stable lines were created (data not shown), whereby five (clones 3′, 5′, 8′, 10′, 11′) lines with variable hnRNPC1 overexpression were further evaluated for 1,25(OH)2D (10 nmol·L−1, 6 h) mediated induction of endogenous mouse (mu) Cyp24a1 mRNA. cPPT, central polypurine tract.
hnRNPC2 subunit and its CLZ domain as a binding ligand partner. In all cases, the negative binding free energy indicated favorable interactions between the C13 and C21 CLZ domains regardless of the species derivation of the subunits. As shown in Table 1, the highest negative binding energy was observed with huC13–huC21, possibly due to

| Gene       | Fold induction |
|------------|----------------|
| Bglap      | 2.0            |
| Cyp24a1    | 1.8            |
| Ddit4      | 1.6            |
| Spp1       | 2.0            |
| Fold induction | 1.0  |

Figure 3. Equimolar expression of human hnRNPC1 and C2 confers resistance to 1,25(OH)2D in mouse MC3T3-E1 osteoblasts. (a) Schematic representation of the mouse col1a1 2.3 kbp promoter-driven piconoviral P2A sequence construct for the co-expression of human (hu) hnRNPC1 and C2 proteins with a GFP reporter. (b) Western blot analyses showing overexpression of hnRNPC1 and C2 proteins using the P2A construct enriched in the nuclear fraction of MC3T3-E1 cells at 96 h post transfection. Empty vector (E), and high (H, 2 μg per well) and low (L, 0.1 μg per well) levels of P2A expression vector cDNA were transfected into MC3T3 cells. (c) 1,25(OH)2D (0.1–10 nmol·L−1; 6 h) induction of mouse osteoblastic genes after transfection of empty vector or hnRNPC1/C2 P2A expression construct in MC3T3-E1 96 h post transfection.

Figure 4. Efficient transfection of the P2A hnRNPC1/C2 construct. (a) Transient transfection of P2A hnRNPC1/C2 construct with IRES-GFP reporter shows efficient labeling of both J774A.1 mouse monocytic and MC3T3-E1 osteoblastic cells after 96 h post transfection. The empty vector control also depicts efficient and comparable GFP labeling. (b) Western analysis of whole cell lysate for beta-actin and hnRNPC1/C2 expression 96 h post transfection.
the additional favorable interactions that could occur between Asn-200 and Glu-186 in the tetramer (Figure 7c, left panel). By contrast, only one hydrogen bond is possible for the muC1−huC2 complex (Figure 7c, right panel), providing a potential explanation for the relatively low negative binding energy for the mouse hnRNPC1/C2 tetramer. The negative free energy of the interspecies oligomeric combinations, muC1−huC2 (−107.22 kcal·mol⁻¹) and huC1−muC2 (−103.25 kcal·mol⁻¹), were intermediate to the species-specific tetramers (Table 1). These results indicate that the Asn-200 in the human hnRNPC CLZ domain plays a pivotal role in stabilization of the hnRNPC1/C2 oligomer in man. To assess the potential impact of hnRNPC subunit binding energy variations on tetramer stabilization, molecular dynamic simulations were carried out (Figure 7d). Data are shown as the root mean square deviations for the backbone atoms (N–CA–C–O) from the experimental structure, over 10 ns for the four tetrameric combinations depicted in Table 1. The huC1−muC2 interspecies tetramer complex showed large fluctuations (instability) during the initial phases of the 10-ns period of simulation, but eventually reached a state comparable the other tetramers after 7 ns.

Figure 5. Cell type-specific action and temporal regulation of the P2A hnRNPC1/C2 construct. (a) Endogenous mouse (mu) type I collagen expression was monitored over a 96-h period under the different transient transfection conditions. For both transfected constructs into MC3T3-E1 cells, col1a1 mRNA expression was delayed when compared to the untransfected controls. In J774A.1 mouse monocytes the expression of endogenous mu type I collagen was variable between the transfected P2A construct and untransfected control, and unregulated in the empty vector control. (b) Human (hu) hnRNPC mRNA levels were monitored in the mouse col1a1 promoter-driven P2A construct-transfected cells at 96 h post transfection. Hu hnRNPC mRNA was temporally regulated in a similar fashion as mu type I collagen in MC3T3-E1 osteoblasts. No temporal regulation of huhnRNPC mRNA was observed in J774A.1 mu monocytes transfected with the P2A construct, suggesting the mu col1a1 2.3 kb promoter was bone-specific. (c) After 96 h of transfection, MC3T3-E1 cells harboring the P2A hnRNPC1/C2 construct express huhnRNPC with mRNA levels approximately 125-fold higher relative to the matching empty vector control when compared to the J774A.1 mouse monocytic cell line.

Figure 6. Endogenous mouse hnRNPC not responsible for functional effects. Endogenous mouse (mu) hnRNPC expression was monitored in MC3T3-E1 cells after transfection and 6-h treatment with various concentrations of 1,25(OH)₂D₃.

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DISCUSSION

The goal of the present study was to develop a mouse model that recapitulated the hnRNPC1/C2 (VDRE-BP) overexpression-induced vitamin D-resistant state observed in vivo in the skeletons of growing nonhuman and human primates. Before developing the animal model, we assessed the effects of hnRNPC1/C2 overexpression using a mouse osteoblast cell line. The studies here show that overexpression of huC1 and huC2 in combination or transfection with a bone-specific, poly-cistronic vector suppressed transspecies 1,25(OH)₂D-mediated induction of mouse osteoblast target gene expression. This finding suggests that human hnRNPC1/C2 can function as a dominant-negative-acting inhibitor.
hnRNPs were first recognized for their ability to bind to single-stranded RNA. However, it was subsequently determined that hnRNPs have the ability to bind both single- and double-stranded DNA with specific cis-acting DNA sequences being important determinants in transcriptional regulation of gene expression under the control of steroid hormone receptors such as VDR and the estrogen receptor. In the current study, overexpression of either huC1 or huC2 in human osteoblasts was sufficient to suppress 1,25(OH)2D-VDR-mediated signaling. Unexpectedly, repression of 1,25(OH)2D-VDR-directed transcription was only achieved by co-overexpression of both huC1 and huC2 in murine osteoblasts. These data, together with molecular dynamic simulation analyses presented in Figure 7, suggest that optimal DNA binding and the subsequent dominant-negative action of hnRNPC1/C2 on transcription occurs with species-specific tetramerization patterns involving the C1 and C2 isoforms (Figure 7). Binding free energy modeling further suggests that this differential response is due to subtle variations in the protein–protein interactions required for hnRNPC1/C2 tetramerization.

While the computational molecular dynamic studies presented here showed that interspecies tetramers of C1 and C2 can form (Figure 7), it is also known that tetramer stability is influenced by elements in the negatively charged CTD of hnRNPC that have diverged most between mouse and man. For example, a deletion construct of the human hnRNPC lacking the CTD beyond the leucine zipper can exist as a dimer, highlighting the importance of this region of hnRNPC protein for tetramer assembly. Further, velocity sedimentation analysis of deletion constructs of the hnRNPC CTD demonstrates that the residues between S-240 and G-263 play a critical role in binding to pre-mRNA and snRNA with high affinity. Based on detailed sequence-specific labeling and solution structural analyses, the current model suggests that hnRNPC proteins exist in an anti-parallel four-helix tetrameric bundle which is centrally positioned and that these bundles assemble through highly stable associations of their leucine zipper CLZ domains. It is the basic leucine zipper-like motif domain, rather than the RNA recognition motif, which plays the major role in binding to pre-mRNA and snRNA with high affinity. Based on detailed sequence-specific labeling and solution structural analyses, the current model suggests that hnRNPC proteins exist in an anti-parallel four-helix tetrameric bundle which is centrally positioned and that these bundles assemble through highly stable associations of their leucine zipper CLZ domains.

The human and mouse CLZ domains are well conserved with the only difference being an Asp to Ser change at the terminal helix of the coiled coil structure (Figure 7b). The CTD is thought to be involved in protein–protein interactions that serve to increase specificity of nucleotide binding. Although there is increasing awareness of the composition, function and mechanisms associated with hnRNPC and homologous proteins, the protein–protein interactions that participate in their recruitment to cis elements along genomic DNA have yet to be fully defined. Emerging evidence shows that the CTD of hnRNPs interacts with transcription elongation factors, chromatin-modifying complexes and pre-mRNA maturation enzymes, thus providing a molecular basis for the coupling between gene transcription and RNA processing. While the hnRNPC protein domain responsible for interaction in cis with VDREs regulated expression of 1,25(OH)2D-driven target genes remains uncertain, our modeling indicates that the DNA transbinding domain may be located within a hinge portion of the acidic CTD. This would place it apart from the core bundle stabilization domain known to bind cis elements which vary among higher and lower mammalian species.
in mind, we postulate that changes in the helix-interaction structures of hnRNPC tetramers can fundamentally alter its binding affinity for cis elements controlling 1,25(OH)₂D–VDR-driven target genes.

It is clear that the full range of hnRNPC activities has not been elucidated. For example, members of the hnRNPC family can bind single-stranded DNA,⁴⁰ suggesting that they may have distinct roles in DNA replication and recombination events as well as in transcription and transcript handling. The identification of species-specific cellular functions of hnRNPC proteins and their specific DNA targets will greatly help to elucidate the broader role of this versatile nuclear protein, in particular its role in vitamin D physiology and diseases that are marked by relative degrees of target tissue resistance to active vitamin D metabolites.

Conflict of Interest
The authors have no financial relationships to disclose and no conflicts of interest.

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