Functional Characterization of Heterogeneous Nuclear Ribonucleoprotein C1/C2 in Vitamin D Resistance

A NOVEL RESPONSE ELEMENT-BINDING PROTEIN

Hong Chen, Martin Hewison, and John S. Adams

From the Division of Endocrinology, Diabetes and Metabolism, Burns and Allen Research Institute, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, California 90048

Clinically apparent hereditary vitamin D-resistant rickets (HVDRR) usually results from a loss of function mutation in the vitamin D receptor (VDR). We recently described a human with the classical HVDRR phenotype but normal VDR function. Hormone resistance resulted from constitutive overexpression of heterogeneous nuclear ribonucleoprotein (hnRNP) that competed with a normally functioning VDR-retinoid X receptor (RXR) dimer for binding to the vitamin D response element (VDRE). Here we describe the purification, molecular cloning, and expression of this vitamin D resistance-causing, competitive response element-binding protein (REBiP) hnRNP C1/C2. When overexpressed in vitamin D-responsive cells, cDNAs for both hnRNPC1 and hnRNPC2 inhibited VDR-VDRE-directed transactivation (28 and 43%, respectively; both \( p < 0.005 \)). By contrast, transient expression of an hnRNP C1/C2 small interfering RNA increased VDR transactivation by 39% \( (p < 0.005) \). Chromatin immunoprecipitation of nucleoproteins bound to the transcriptionally active 1,25-dihydroxy vitamin D3 (1,25(OH)\(_2\)D3)-responsive element (11, 12). And sixth are the “co-modulator” Cis-acting proteins of the heterogeneous nuclear ribonucleoprotein (hnRNP) family that compete with the VDR-RXR for binding to the vitamin D response element (VDRE), thus altering hormone receptor-directed transactivation (13–15).

The hnRNPs, first recognized for their ability to bind single strand ribonucleotides (16, 17), are a family of more than 20 proteins that contribute to the complex associated with nascent pre-mRNA and are thus able to modulate RNA processing, including the stabilization of pre-mRNAs for nuclear export and translation (18–20). More recently, hnRNPs have been shown to be capable of binding DNA in both single-stranded (16, 17) and double-stranded formats (14, 15). Acting in their capacity as double-stranded DNA-binding proteins, we have shown previously that individual hnRNPs may function as dominant-negative modifiers of steroid hormone-mediated transcription by competing with the VDR-RXR (14, 15) and the estrogen receptor (21–23) for VDRE and estrogen response element, respectively. Here we describe the isolation, purification, and cloning of the cDNA for the naturally occurring human retinoid X and vitamin D response element-binding protein (REBiP), which was shown to be hnRNP C1/C2. In further studies, we have demonstrated the ability of the REBiP to exert a dominant-negative effect on 1,25-dihydroxy vitamin D3 (1,25(OH)\(_2\)D3)-induced transcription via the naturally occurring VDRE in the response element-binding protein; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation; FCS, fetal calf serum; ROS, rat osteosarcoma; Pipes, 1,4-piperazinediethanesulfonic acid.
vitamin D-24-hydroxylase (CYP24) gene promoter. Finally, using chromatin immunoprecipitation (ChIP) we have revealed the temporal pre-existence of the REBiP on the VDRE in vivo in cells in advance of VDRE occupation by the liganded VDR, suggesting a possible role for the REBiP promoting cis-site-specific chromatin remodeling in the region of the human genome harboring VDREs.

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture—Crystalline 1,25(OH)2D3 (Biomol, Plymouth Meeting, PA) was solubilized in 100% ethanol for addition to reaction mixtures. An Epstein-Barr virus transformed B-lymphoblast cell line and primary dermal fibroblast cultures from a VDR-RXR-normal patient with classical symptoms of hereditary vitamin D-resistant rickets (HVDRR) were used as a source for REBiP (15). Cultures of cells from an unrelated female of the same age with normal skeletal development were used as control cells for comparison. Immortalized B-cells were routinely maintained in RPMI 1640 medium (Invitrogen) supplemented with 5% fetal calf serum (FCS) (Omega, Tarzana, CA). VDR-VDRE-reporter assays were performed in the VDRE + ROS (rat osteosarcoma) 17/2.8 cells, a cell line stably transfected with luciferase under the control of the VDRE in the CYP24 promoter (a kind gift from Hector De Luca, University of Wisconsin, Madison, WI) as previously described (24). VDRE + ROS cells were propagated in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 6 mM glutamine, 10 μg/ml insulin, and 10% FCS. Vitamin D-responsive human kidney HKC-8 cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 media supplemented with 5% FCS.

Purification and Molecular Cloning of REBiP—A DNA affinity resin was prepared as described by Kadonga and Tijan (25). Gel-purified oligonucleotides (30-mers) containing nucleotides of the complementary sequence to consensus RXRE (5’-GATCAGCTTCAGGTCA GAGGTCAGAGAGCT-3) with 4-bp cohesive ends were annealed with their complementary sequence, subjected to 5’-phosphorylation, and then concatamerized in reactions using DNA ligase. The concatamerized DNA was coupled to cyanogen bromide-activated Sepharose. Affinity-purified response element-binding proteins present in the nuclear extracts of HVDRR cells were sequentially eluted from the support in increasing concentrations of KCl as described previously (21). Selected fractions were then submitted to automated Edman degradation and amino acid sequencing using an Applied Biosystems 477A or Hewlett Packard G1005 protein sequencer (Harvard Microchemistry Facility, Cambridge, MA) as previously described (15).

Following the identification of hnRNP C1/C2 as the REBiP from amino acid sequencing, a cDNA expression construct, including the coding region of the hnRNP C1/C2 gene, was cloned. Approximately 200 ng of HVDRR total RNA, isolated with TRIzol reagent (Invitrogen), was used as a template. Successful amplification of the coding region cDNA sequence was achieved with 30 cycles of reverse transcription-PCR using the primers to hnRNP C1/C2 (5’-ACGATGGCCAGCAACGT-TACCAAC-3’ and 5’-TCCTCCATTGGCGCTGTCCT-3’). The resulting PCR product was ligated into the PCR 3.1/V5-His-TOPO vector (Invitrogen) and the cDNA sequence verified.

Antibodies and Immunoblotting Assays—Cell extracts from the HVDRR patient and the control subject were subjected to electrophoresis on 4–20% SDS-PAGE and transferred to nitrocellulose membranes. Western blot analyses, using a panel of antibodies including anti-human hnRNP C1/C2, anti-human hnRNPC-like, anti-human hnRNPA, anti-human Ku protein, and anti-human actin antibody (all from Santa Cruz Biotechnology, Santa Cruz, CA) were performed as described previously (14, 15, 21, 22).

Transient Transfection and Small Interfering RNA (siRNA) Assays—Cells were grown to 80–90% confluency in 12-well plates. For transient expression analyses, each well received 0.8 μg of REBiP cDNA expression plasmid or vector-alone plasmid. For siRNA experiments, each well received 10 μM siRNP C1/C2 (Santa Cruz Biotechnology). Promoter-reporter assays carried out using VDRE + ROS cells utilized the endogenous luciferase construct, whereas similar assays using HKC-8 cells required transfection of 0.8 μg of the VDRE-luciferase plasmid. All transfections were carried out in 0.004% Lipofectamine-2000 in Opti-MEM (Invitrogen) followed by an overnight incubation. The next day, the medium was replaced by Opti-MEM containing 0.1% ethanol or 10 nm 1,25(OH)2D3. After an additional 24 h at 37 °C, the cells were lysed and luciferase and β-galactosidase activities measured (Promega, Madison, WI).

Electrophoretic Mobility Shift Assay and ChIP Assays—Electrophoretic mobility shift assays were performed as described previously (15) using REBiP-enriched nuclear extract from DNA affinity chromatography as a source of response element-binding proteins.

ChIP assays were performed as described previously (26). Briefly, HVDRR and control lymphocytes were cultured for 4 days in RPMI 1640 medium supplemented with 5% charcoal-stripped, heat-inactivated FCS and treated with 1,25(OH)2D3 for the indicated times. Following hormone treatment, the cells were washed twice with phosphate-buffered saline and cross-linked with 1% formaldehyde at 37 °C for 10 min. After quenching of the cross-linking with 1.25 M glycine, cells were harvested, rinsed with phosphate-buffered saline, and the resulting pellets were resuspended in 1 ml of cell lysis buffer (5 mM Pipes, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of peptatin, leupeptin, and aprotinin). Nuclei were collected and resuspended in 500 μl of nuclear lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS, 1 mM dithiothreitol, 2.5 mM phenylmethylsulfonyl fluoride) and 1 μg/ml each peptatin, leupeptin, and aprotinin (all from Sigma). The resulting chromatin samples were sonicated to yield sheared DNA fragments of sizes between 300 and 1000 bp. For each immunoprecipitation, sheared chromatin was diluted with immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, and 167 mM NaCl). The chromatin was collected and incubated at 4 °C overnight with 5 μg of anti-VDR-9A7 (Affinity Bioreagents Inc. Golden, CO) and anti-hnRNP antibodies (Santa Cruz Biotechnology). Rabbit IgG was used as a negative control. The immune complexes were precipitated with 60 μl of protein A-Sepharose beads (source at DECEMBER 22, 2006 • VOLUME 281 • NUMBER 51

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4 °C for 1 h. The beads were then subjected to serial 1-ml washes of the following: immunoprecipitation dilution buffer TSE-500 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl); LiCl/detergent buffer (100 mM Tris-HCl, pH 8.1; 500 mM LiCl; 1% Nonidet P-40; and 1% deoxycholic acid in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The antibody-protein-DNA immunocomplexes were then eluted with 1% SDS in 50 mM NaHCO₃. Formaldehyde cross-linking was reversed by heating at 65 °C overnight with the addition of 5M NaCl to a final concentration of 200 mM. All of the samples were then digested at 45 °C for 1 h with 20 μg of proteinase K. DNA was extracted by phenol-chloroform, ethanol-precipitated, and analyzed by PCR using amplifying primer sequences spanning the enhancer VDRE in the proximal CYP24 promoter, bp 334 to 22 bp relative to the start site of transcription. Amplifying primer pairs spanning a more distal section of the CYP24 promoter not harboring a VDRE, bp 1749 to 1424, were used as a negative control in ChIP assays.

RESULTS

Purification and Molecular Cloning of the REBiP—Previous work from this laboratory (14) identified two species of an anti-hnRNP-reactive protein from vitamin D-resistant New World primate nuclear extracts that bound specifically to a consensus human VDRE (VDRE-D3, AGGGTCAGACAGGTCA) direct repeat motif in both double- and single-stranded format. Subsequently, we screened human HVDRR cell extracts for their ability to bind to the same response elements in vitro and determined that, although both cis elements could specifically bind HVDRR extract, the most avid interaction appeared to be with the RXRE in double-stranded DNA format (15). Given these preliminary results, we used concatamers of the double-stranded RXRE as an affinity agent to purify the human REBiP. The affinity support bound a nuclear protein with a tryptic fragment bearing 100% sequence identity with the human hnRNPC1 and C2 proteins (Fig. 1A), the alternatively spliced, translated products of the hnRNPC1/C2 gene. Using tryptic peptide sequencing, a full-length REBiP cDNA was cloned that bore 99.5% nucleotide sequence identity and 99.3–99.7% deduced amino acid sequence identity with the human hnRNPC1 and C2 proteins. Western blot analyses using an anti-human hnRNPC1/C2 antibody confirmed that cells from the HVDRR patient overexpressed a pair of anti-hnRNPC1/C2-reactive proteins of 39–40 kDa, compatible with the hnRNPC1 and the slightly larger hnRNPC2 (Fig. 1B). This contrasted with extracts from an age- and sex-matched normal, vitamin D-sensitive donor that expressed principally hnRNPC2. Data in Fig. 1C showed that the endogenous hnRNPC1/C2 gene product was not confined to the nucleus, as the REBiP was also readily detectable in the post-nuclear supernatant of both HVDRR and control lymphocytes.

REBiP Specifically Interacts with the VDRE and Blocks VDRE-directed Transactivation—Confirmation of the ability of the affinity-purified REBiP or hnRNPC to bind to a VDRE and affect VDRE-directed transactivation was then sought. As was
the case with crude extracts from HVDRR cells (15), affinity-purified REBiP was specifically bound to the VDRE in double-stranded format (Fig. 2B) and competed with 100× unlabeled oligonucleotide for occupation of a VDRE bearing a direct repeat of the AGGTCA (VDRE-D3) or RXRE (Fig. 2A). These data suggested that the affinity-purified hnRNPC could bind specifically to a direct repeat of AGGTCA separated by either one or three base pairs and might function in a dominant-negative mode to block VDRE-directed transactivation. Promoter-reporter analyses confirmed that transient overexpression of the unspliced hnRNPC1/C2 cDNA in wild-type, vitamin D-sensitive cells blocked VDR-VDRE-directed reporter activity in the absence or presence of added 1,25(OH)2D3 (57 and 44% decrease, respectively, compared with vector-only control; both p < 0.005) (Fig. 3A). Somewhat unexpectedly, co-expression of the siRNA for hnRNPC1/C2 increased transcription both in the absence and presence of stimulatory 1,25(OH)2D3 (39 and 135% increase, respectively, compared with vector only control; both p < 0.005). These data suggested that an endogenous, functional, human hnRNPC1/C2 siRNA-reactive mRNA for a REBiP is normally expressed in this rat cell line; the inset Western blot (Fig. 3B) confirms this to be the case. Data in Fig. 3C shows that overexpression of hnRNPC1 and hnRNPC2 cDNAs alone or together suppressed VDR-VDRE-directed reporter activity in the absence or presence of added 1,25(OH)2D3. This inhibitory effect on transcription appeared to be modulated by a combination of the two splice variants, with hnRNPC2 being slightly more potent than hnRNPC1 and the inhibitory effect of the two cDNAs being additive in the absence of 1,25(OH)2D3.

REBiP Occupies the VDRE in Vivo—To determine whether the hnRNCP1/C2 REBiP could affect 1,25(OH)2D3-stimulated transcription in living cells, we performed ChIP assays looking for proteins from vitamin D-resistant HVDRR and vitamin D-responsive cells that bound to the VDRE in double-stranded format. Data in Fig. 3B show that overexpression of hnRNPC1 and hnRNPC2 cDNAs alone or together suppressed VDR-VDRE-directed reporter activity in the absence or presence of added 1,25(OH)2D3. This inhibitory effect on transcription appeared to be modulated by a combination of the two splice variants, with hnRNPC2 being slightly more potent than hnRNPC1 and the inhibitory effect of the two cDNAs being additive in the absence of 1,25(OH)2D3.

FIGURE 2. REBiP specifically binds to VDRE and RXRE. Nuclear extracts (NE) from the HVDRR cells binds double-stranded RXRE (A) and VDRE-D3 (B). Binding was also displaced by a 100-fold excess of unlabeled RXRE (A, lane 3) or VDRE-D3 (B, lane 3) and by anti-hnRNCP1/C2 antibody (A and B, lanes 4).

FIGURE 3. Altered expression of hnRNCP1 and C2 modulates VDRE-directed transactivation. A, VDRE ROS 17/28 cells with constitutive expression of a CYP24 gene reporter construct were transiently transfected with either an hnRNCP1/C2 expression construct or siRNA to hnRNCP1/C2 in the absence or presence of added 1,25(OH)2D3. Data are the mean of three separate transfections. B, Western blot analysis of hnRNCP1/C2 in the cytoplasmic (Cyto) and nuclear (NE) extracts from HVDRR and ROS 28/1.7 cells (ROS) in the absence or presence of 10 nM 1,25(OH)2D3. C, expression constructs containing cDNA for hnRNPC1 and/or hnRNPC2 were transiently co-transfected into the human kidney HKC-8 cell line in the absence or presence of 10 nM 1,25(OH)2D3. Data are the mean of triplicate determinations of luciferase activity; p < 0.005; #, statistically different from individual hnRNPC1 or C2 treatments; p < 0.001.
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FIGURE 4. REBiP occupies the VDRE in control and HVDRR cells. Primers for VDR non-responsive (A) and responsive (B) regions of the rat CYP24 promoter were used in ChIP assays with antibodies against VDR, hnRNP C1/C2, and normal IgG (as negative control) on cross-linked chromatin isolated from control or HVDRR cell lines treated with either vehicle (0.1% ethanol) 1,25(OH)2D3 (10 nM) for 15 min. The immunoprecipitated DNA was amplified using oligonucleotides outlined in the schematic illustration of the promoter in the upper panel, and amplified ChIP fragments were identified by gel electrophoresis as shown in the lower panel.

FIGURE 5. Analysis of temporal changes in the association of VDR and hnRNP C1/C2 with rat CYP24 promoter. ChIP analysis of VDR, hnRNP C1/C2, or IgG (as negative control) on the native rat CYP24 promoter was carried out for lysates isolated from control or HVDRR cells treated with or without 1,25(OH)2D3 (10 nM) for different periods from 0 to 165 min. The associated chromatin DNA fragments were amplified with 1,25(OH)2D3-responsive region (−334 to −22 bp) (Fig. 4). By contrast, in vitamin D-responsive human B lymphoblasts, the proximal VDRE-containing promoter region of the CYP24 promoter (−334 to −22 bp) (Fig. 4B) was occupied mostly by anti-human hnRNP C1/C2-reactive protein and just minimally by the VDR in the basal state before 1,25(OH)2D3 treatment. After exposure to a VDR-saturating concentration of 1,25(OH)2D3, the VDR was recruited to the VDRE with a coincident decrease in hnRNP C1/C2 interaction with the promoter. This suggested that there was competition between the two VDRE-binding proteins for the cis sequence. The rabbit IgG control did not detect any other proteins interacting with this region of the promoter, indicating that binding of both the hnRNP C1/C2 and VDR was specific. Surprisingly, in REBiP-overexpressing HVDRR cells, both the VDR and the hnRNP C1/C2 REBiP were present on the promoter in the absence of added hormone, and this pattern of relative occupancy of the VDRE by the two proteins changed little upon acute administration of 1,25(OH)2D3. Bearing in mind that this experiment afforded only a single 15-min “snapshot” of hormone-induced changes in VDR and hnRNP C1/C2, these data suggest that the two proteins remained as co-occupants of the VDRE and/or that one of the two was anchored to the VDRE with the other being bound to the other but not necessarily still binding to DNA.

Analysis of Temporal Changes in Association of VDR and hnRNP C1/C2 with the VDRE by ChIP—To further clarify the dynamics of VDRE occupancy, we analyzed temporal changes in the interaction of the hnRNP C1/C2 REBiP with the −334 to −22 bp fragment of the CYP24 gene promoter (Fig. 5). It is now well recognized that occupancy of the VDRE by the VDR-RXR is cyclical in nature, eventually resulting in the relative stable association of the heterodimer with the promoter and sustained transcriptional effects (26). However, the molecular nature of the “on-off” behavior of the VDR-RXR at the VDRE remains a matter of conjecture. We postulated that these receptor-response element cycling events may be due to the competitive presence of the REBiP at the VDRE. As such, we examined the temporal sequence of proteins occupying the VDRE in control vitamin D-responsive and vitamin D-resistant HVDRR cells over a period of 165 min (Fig. 5). As was the case with the ChIP assay data presented in Fig. 4, before the addition of hormone, the VDR was relatively excluded from the VDRE in control vitamin D-responsive cells compared with vitamin D-resistant HVDRR cells in which both the VDR- and anti-hnRNP C1/C2-reactive REBiP were present on the VDRE. Following the addition of 1,25(OH)2D3 to the control cells, the VDR was recruited to the VDRE and the REBiP content diminished. Further, as previously reported by others (26), the VDR cycled on and off the promoter at roughly 45-min intervals. Interestingly, REBiP displayed a reciprocal cycling pattern occupying the VDRE every 45 min when the VDR was off the cis element, with relatively less of the hnRNP C1/C2 gene product occupying the VDRE in favor of the VDR at the conclusion of the 165-min observation period. These results suggested that the VDR and REBiP were competing for occupancy of the VDRE. In contrast to the reciprocal on/off cycling of the VDR and hnRNP C1/C2 in vitamin-responsive control cells, the cycle time for the VDR with the VDRE in HVDRR cells was 60 min following treatment with 1,25(OH)2D3. Moreover, in HVDRR cells, the cycle “off” time for hnRNP C1/C2 was 45–60 min, not 15 min, after the addition of 1,25(OH)2D3, and the reciprocal association of the VDR and hnRNP C1/C2 with the VDRE was distorted compared with that in control vitamin D-responsive cells.
DISCUSSION

The human *hnRNPC1/C2* gene encodes two alternatively spliced translation products, hnRNPC1 and hnRNPC2 (16); as depicted in Fig. 1A, hnRNPC1 is the smaller of the two variants created by excision of 13 amino acids from the hnRNPC1/C2 coding sequence (27, 28). Along with protein partners hnRNPA1 and -A2 and hnRNPB1 and -B2, hnRNPC1 and -C2 are the core elements of the hnRN complex that is central to the handling of RNA transcripts for intracellular translation or destruction (19, 29, 30). The *hnRNPC1/C2* gene products have also been shown to have additional actions in the nuclear compartment of cells, including splicing, telomere regulation, as well as nuclear retention of hnRNAs and nuclear matrix (31–35). As part of their action in RNA processing, the hnRNPA, -B, and -C proteins form heterotetrameric complexes that assemble as an anti-parallel 4-helix coiled coil (36) on nascent transcripts to regulate the splicing, polyadenylation, and turnover of that transcript (37). Among the core hnRNPA, -B, and -C proteins, hnRNPC1 and C2 were previously considered to be the only members of the grouping not found outside of the nucleus of the cell (38). However, our results (Fig. 1C), along with the work of others (37, 39), demonstrate that *hnRNPC1/C2* gene products can be recovered from the cytoplasm of the cell, with the export signal for hnRNPC1 and -C2 residing in a 40-amino-acid stretch of the C-terminal domain of the protein (37).

Recent work from this laboratory has also confirmed that hnRN binding to nucleic acid is not confined to single strand mRNA but can be observed using either single or double strand DNA as a binding template (14, 21). When overexpressed in vivo, as they are in certain steroid hormone-resistant New World primate genera (7), these proteins can compete with ligand-bound sterol/steroid receptor dimer pairs for binding to that receptor’s cognate cis response element and block ligand-mediated transactivation (23). These New World primate species represent a successful experiment of nature as they have evolved effective means of countering the dominant-negative actions of the hnRNPs as promoter-silencing factors by amplifying the endogenous production of the dominant positive-acting receptor-activating ligands (37). In 2003, we reported the first human example of clinical vitamin D resistance due to the only members of the grouping not found outside of the nucleus of the cell (38). However, our results (Fig. 1C), along with the work of others (37, 39), demonstrate that *hnRNPC1/C2* gene products can be recovered from the cytoplasm of the cell, with the export signal for hnRNPC1 and -C2 residing in a 40-amino-acid stretch of the C-terminal domain of the protein (37).

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As anticipated, based on earlier work characterizing the hormone-resistant phenotype of our HVDRR patient and New World primates, overexpression of the *hnRNPC1/C2* cDNA as well as the *hnRNPC1* cDNA and the *hnRNPC2* cDNA alone or together exerted a dominant-negative effect on 1,25(OH)₂D₃-driven VDRE-promoter activity (Fig. 3). More unexpected was the finding in the same experiments that a significant dominant-negative effect of hnRNPC1/C2 was exerted in the basal, no-hormone-added state in wild-type, vitamin D-sensitive osteosarcoma (ROS 17/2.8) and kidney (HKC-8) cells (Fig. 3, A and C), suggesting that “hormone-sensitive” promoters such as that found in the CYP24 gene can be extraordinarily susceptible to the dampening effects on transcription when only a small amount of 1,25(OH)₂D₃ is available (i.e. as in diluted FCS) to the endogenous VDR. Even more unexpected was the significant stimulatory effect of hnRNPC1/C2 siRNA on VDRE-reporter activity in ROS 17/2.8 cells (Fig. 3A), suggesting that the “knockdown” of endogenously expressed hnRNPC1/C2 in vitamin D-responsive cells can relieve a naturally occurring brake on transcription. These results further indicate that the dominant-negative effects of endogenous hnRNPC1/C2 cannot be “competed out” completely by the addition of exogenous 1,25(OH)₂D₃. In fact, the presence of the hnRNPC1/C2 REBiP on the VDRE in vitamin D-responsive as well as HVDRR cells subjected to ChIP (Fig. 4B) confirms that the REBiP is bound to the VDRE in vivo.

Because of its relative abundance in the HVDRR compared with control nuclei (Fig. 1C), it is perhaps not surprising to find relatively more of the hnRNPC1/C2 REBiP occupying the VDRE in the basal state (Fig. 4B). However, it was somewhat unexpected to detect the presence of REBiP on the VDRE in vitamin D-responsive control cells in the basal state (Figs. 4 and 5). These data suggest that hnRNPC1/C2 is normally bound by VDRE-like cis elements preceding occupation of that cis sequence by its cognate receptor. This observation and the ability of the REBiP to bind single strand DNA with an AGGTCA motif in a direct repeat format (15) have led us to speculate that hnRNPC1/C2, and not the receptor protein itself as previously suggested (41), initiates the program of chromatin remodeling. Such hnRNPC1/C2 “priming” for chromatin remodeling has been demonstrated recently for an enhancer element in the β-globin promoter (42). If this is the case, then one would predict that the introduction of ligand 1,25(OH)₂D₃ would promote more effective binding of the liganded VDR-RXR dimer pair to VDRE. This appears to be the case. In wild-type, vitamin D-responsive cells, the addition of 1,25(OH)₂D₃ to the ChIP reactions results in recruitment of the VDR to the VDRE. There was no discernable change in hnRNPC1/C2 occupancy of the promoter in these non-quantitative experiments; a dynamic range in the number of rounds of PCR amplification to which ChIP fragments have been exposed has not yet been performed to assess the relative robustness of hnRNPC1/C2 binding to the VDRE before and after the addition of 1,25(OH)₂D₃.

Data presented in this study provide further evidence that, in addition to their established function as facilitators of post-transcriptional gene regulation, hnRNPs are able to influence
gene transcription itself by acting as binding proteins for hormone response elements (REBiP). We have shown that the REBiP associated with VDR-mediated transactivation is hnRNP C1/C2 and confirm that overexpression of this factor leads to suppression of vitamin D-mediated gene regulation in a similar fashion to that originally described for a patient with HVDRR (15). Significantly, the REBiP function of hnRNP C1/C2 was also evident in normal vitamin D-responsive cells, where its occupancy of the CYP24 VDRE was reciprocal to the VDR. It is therefore possible to hypothesize that REBiPs play an important role in directing the cyclical on-off equilibrium between the VDR and its target CYP24 response element (43). Moreover, the identification of an REBiP associated with estrogen receptor-mediated gene regulation suggests that this may be a common determinant of temporal variations in response element occupancy by steroid hormone receptors (22). As well as clarifying the general applicability of REBiP-receptor interaction, future studies will be required to elucidate the putative function of hnRNPs as initiators of chromatin remodeling and the potential link between this and the more established post-transcriptional function of hnRNPs.

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