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All-*trans*-retinoic Acid Increases *SLC26A3* DRA (Down-regulated in Adenoma) Expression in Intestinal Epithelial Cells via *HNF-1β*[*]

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Background: Down-regulation of Cl^-/HCO_3^- exchanger *SLC26A3* in gut inflammation results in diarrhea. Does all-*trans*-retinoic acid (ATRA), an anti-inflammatory agent, affect *SLC26A3* (DRA)?
Results: ATRA increases DRA expression in enterocytes via transcriptional activation through *RAR-β*/*HNF-1β* signaling.
Conclusion: ATRA up-regulates DRA expression.
Significance: ATRA may act as a therapeutic agent for diarrhea by augmenting DRA expression.

All-*trans*-retinoic acid (ATRA) is an active vitamin A derivative known to modulate a number of physiological processes, including growth and development, differentiation, and gene transcription. The protective effect of ATRA in gut inflammation and diarrheal diseases has been documented. In this regard, down-regulated in adenoma (DRA, a key luminal membrane Cl^- transporter involved in NaCl absorption) has been shown to be suppressed in intestinal inflammation. This suppression of DRA is associated with diarrheal phenotype. Therefore, current studies were undertaken to examine the effects of ATRA on DRA expression. DRA mRNA levels were significantly elevated (~4-fold) in response to ATRA with induction starting as early as 8 h of incubation. Similarly, ATRA increased DRA protein expression by ~50%. Furthermore, DRA promoter activity was significantly increased in response to ATRA indicating transcriptional activation. ATRA effects on DRA expression appeared to be mediated via the *RAR-β* receptor subtype, as ATRA remarkably induced *RAR-β* mRNA levels, whereas *RAR-β* knockdown substantially attenuated the ability of ATRA to increase DRA expression. Results obtained from agonist (CH-55) and antagonist (LE-135) studies further confirmed that ATRA exerts its effects through *RAR-β*. Furthermore, ATRA treatment resulted in a significant increase in *HNF-1β* mRNA levels. The ability of ATRA to induce DRA expression was inhibited in the presence of *HNF-1β* siRNA indicative of its involvement in ATRA-induced effects on DRA expression. In conclusion, ATRA may act as an antidiarrheal agent by increasing DRA expression via the *RAR-β*/*HNF-1β*-dependent pathway.

All-*trans*-retinoic acid (ATRA),[2] a major active metabolite of vitamin A, is known to regulate several biological processes. For example, ATRA signaling has been shown to be important for inducing cellular differentiation, growth, and development of epithelial cells in various tissues including intestine (1–3). Also, a role of vitamin A and its metabolites in maintenance of intestinal epithelial integrity (4, 5) and immune homeostasis (6) has been established. Other beneficial effects of ATRA on intestinal mucosa include attenuation of intestinal inflammation and injury in neonatal rat model of necrotizing enterocolitis (7), 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis (8), and human ulcerative colitis (9). Effect of vitamin A supplementation in reducing severity of diarrheal episodes and diarrhea-associated infant mortality has been reported (10, 11). Furthermore, substantial contribution of vitamin A in preventing diarrhea in children with HIV infection or exposure to maternal HIV infection is also documented (12). Reduced incidences of respiratory infection and diarrhea-associated mortality are observed in children supplemented with weekly low doses of vitamin A (13) or iron and vitamin A co-supplementation (14). Repletion therapy of this vitamin in deficient individuals has been shown to reduce the risk of diarrhea and gut-barrier dysfunction emphasizing the anti-diarrheal nature of this micronutrient (15). However, the mechanisms underlying the anti-diarrheal effects of vitamin A are not understood.

Diarrhea is the most debilitating symptom associated with enteric infections or intestinal inflammation (16, 17). Although multifactorial in nature, diarrhea usually occurs when there is increased fluid and electrolyte secretion and/or reduced absorption. In this regard, electroneutral NaCl absorption in the ileum and colon predominantly occurs via the coupled operation of apical Na^+/H^+ exchanger (NHE3) and Cl^-/HCO_3^- exchanger *SLC26A3* or DRA (down-regulated in adenoma). Several lines of data implicate dysregulation of DRA in pathophysiology of diarrheal disorders. For example, mutations

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[2] The abbreviations used are: ATRA, all-*trans*-retinoic acid; RXR, retinoic X receptor; DRA, down-regulated in adenoma; RAR, retinoic acid receptor; HNF, hepatocyte nuclear factor.

TABLE 1
Primers used for real-time PCR

Gene	Species	Accession No.	Primer sequence
SLC26A3 or DRA	Human	BC025671	(F) 5′-TTCAGTTGCCAGCGTCTATTC-3′ (R) 5′-GTGTTTTGCCTCCTGTGCTCT-3′
SLC26A6 or PAT-1	Human	NM_022911	(F) 5′-AGATGCCCCACTACTCTGTCCT-3′ (R) 5′-ATCCACACCACACCTCTGCTT-3′
GAPDH	Human	NM_001101.3	(F) 5′-GAAATCCCATCACCATCTTCC-3′ (R) 5′-AAATGAGCCCCAGCCTTCT-3′
HNF-1α	Human	NM_000545	(F) 5′- TCCTGTATTTGTTCCCAAGAGCATC-3′ (R) 5′-TCCCACAGGAGTAAGGACGACTTC-3′
HNF-1β	Human	NM_000458	(F) 5′-CCAAGCCGGTCTCCATACTC- 3′ (R) 5′-TGGGAGGTGTGTCATAGTCGT-3′
HNF-4α	Human	NM_175914	(F) 5′-GTTCAAGGACGTGCTGCTCCTA-3′ (R) 5′-ATGGACACCCGGCTCATCTC-3′
RAR-α	Human	NM_001145301	(F) 5′-GGGCAAATACACTACGAACAACA-3′ (R) 5′-CTCCACAGTCTTAATGATGCACT-3′
RAR-β	Human	NM_000965	(F) 5′-TGGTGTCTTGTTCTGGGGTAT-3′ (R) 5′-CGTGGAGTTTGCTAAACGTCT-3′
RAR-γ	Human	NM_001243732	(F) 5′-ATGCTGCGTATCTGCACAAG-3′ (R) 5′-AGGCAAAGACAAGGTCTGTGA-3′

in the DRA gene cause congenital chloride diarrhea, characterized by a high volume of watery diarrhea with a massive loss of chloride (18, 19). Also, diarrheal phenotype due to loss of luminal membrane Cl^-/base exchange activity is the predominant feature exhibited by DRA (but not PAT-1) knock-out mice (20, 21). The importance of DRA in diarrheal disorders is further evident from studies demonstrating reduction in DRA expression in animal models of inflammatory and infectious diarrhea and in inflammatory bowel disease patients (22–24). Thus, agents that increase DRA function and expression can be utilized as potential anti-diarrheals. Indeed, previous studies from our laboratory have shown that DRA activity and expression is up-regulated by various anti-diarrheal agents such as the probiotic *Lactobacillus acidophilus* (LA) and the bioactive lipid lysophosphatidic acid. *L. acidophilus* and lysophosphatidic acid-mediated increase in apical Cl^-/HCO_3^- exchange activity occurred via an increase in surface DRA levels as well as via increasing total cellular levels of DRA by transcriptional mechanisms (25–28). The role of nuclear transcription factors *HNF-1*α and *-1*β in regulating DRA expression has also been recently reported (29).

ATRA is known to mediate its effects by binding to nuclear receptors: retinoic acid receptors (*RAR*s-α, -β, and -γ) and retinoic X receptors (*RXR*s-α, -β, and -γ) (30, 31). ATRA binds *RAR* that dimerizes with *RXR* to activate gene transcription. In view of the beneficial effects of ATRA in reducing diarrheal episodes, it was of interest to systematically study the effects of ATRA on DRA expression in intestinal epithelial cells and to elucidate the underlying molecular mechanisms. Utilizing Caco-2 cells as an *in vitro* model, our results demonstrated that ATRA stimulated DRA expression and promoter activity via *RAR-*β. This increase in DRA expression in response to ATRA was mediated via the involvement of transcription factor *HNF-1*β. These findings indicate that ATRA may have potential antidiarrheal effects and may be of benefit as a therapeutic target in the treatment of diarrhea associated with inflammatory or infectious disorders of the gut.

Experimental Procedures

Materials—All-*trans*-retinoic acid, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid were purchased from Sigma. ATRA was dissolved in 100% alcohol to a 10 mM stock solution and stored in the dark at −80 °C. Agonist and antagonist for *RAR-*β, CH-55, and LE-135 and for *RXR*, DHA, and HX-531, respectively, were obtained from Tocris Bioscience (Bristol, UK). Goat anti-rabbit antibody conjugated to horseradish peroxidase was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The luciferase assay system was procured from Promega (Madison, WI) and β-galactosidase assay kit was obtained from Clontech (Palo Alto, CA). All other chemicals were at least reagent grade and were obtained from either Sigma or Fisher Scientific (Pittsburgh, PA).

Cell Culture—Caco-2 cells and Eagle's minimum essential medium were obtained from ATCC (American Type Culture Collection, Manassas, VA). Cells were grown routinely in Eagle's minimum essential medium supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 mg/liter of gentamicin, and 20% fetal bovine serum in 5% CO_2, 95% air environment at 37 °C in T-150-cm^2 plastic flasks. Cells between passages 25 and 45 were used for the present study. Caco-2 cells were plated on 24-well plates (Costar, Corning, NY) at a density of 2×10^4 cells/well. Fully differentiated Caco-2 monolayers (10–14 days post-plating) were treated with ATRA for 8–24 h in serum-free cell culture medium for assessment of DRA mRNA and protein expression. For the promoter studies, 1.30×10^7 cells/well were plated on a 24-well plate and transiently transfected by electroporation utilizing the Amaxa nucleofactor system while still in suspension. Cell monolayers grown on transwell inserts at a density of 4×10^3 cells/well were used for immunofluorescence staining at 11 days post-plating.

RNA Extraction and Quantitative Real-time PCR—Total RNA was extracted from control and treated Caco-2 cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Equal amounts of RNA from both treated and control samples was reverse transcribed and amplified in a one-step reaction using Brilliant SYBR Green qRT-PCR Master Mix Kit (Agilent Technologies, Santa Clara, CA). Gene-specific primers used are listed in Table 1.

Cell Lysates and Western Blotting—Caco-2 cells were treated with 10 μM ATRA for different time periods (8, 16, and 24 h) or with *RAR-*β agonist, CH-55 (1 μM), and antagonist, LE-135 (1

μM) for 24 h. Control cells were treated with vehicle (100% alcohol) at 0.1% final concentration. After treatment, control or treated cells were washed with ice-cold 1× PBS to remove residual media. Total protein was extracted by suspending the cell pellet in cell lysis buffer (Cell Signaling, Danvers, MA) supplemented with protease inhibitor mixture from Roche Applied Science. The cells were lysed by sonication (three pulses for 20 s each) and the lysate was centrifuged at 13,000 rpm for 7 min at 4 °C to remove cell debris. The supernatant containing the total cell proteins was collected and the protein concentration was determined by the Bradford method (32). To examine the expression levels of DRA, equal amounts (75 μg/sample) of whole cell lysates were solubilized in SDS-gel loading buffer and boiled for 5 min. Proteins were loaded on a 7.5% SDS-polyacrylamide gel and transblotted to nitrocellulose membrane after electrophoretic separation. After 1 h of incubation in blocking buffer (1× PBS and 5% nonfat dry milk) the membrane was probed with affinity purified anti-DRA antibody (1:100 dilution). DRA antibody was raised against the C-terminal amino acid (745–764) sequence: INTNGGLRNRVYEPVETKF of *SLC26A3* (accession number: BC025671) (at the Research Resource Centre, University of Illinois at Chicago), or *GAPDH* antibody (Sigma; 1:3,000 dilution) in 1× PBS and 2.5% nonfat dry milk overnight at 4 °C. The membrane was washed five times with wash buffer (1× PBS and 0.1% Tween 20) for 5 min and probed with HRP-conjugated goat anti-rabbit antibody (1:2,000 dilution) for 1 h followed by ECL (enhanced chemiluminescence, from Bio-Rad) detection. The expression of *HNF-1β* and *RAR-β* after siRNA transfection was probed using anti-*HNF-1β* antibody (Santa Cruz, 1:100 dilution) and *RAR-β* antibody (Abcam, 1:200 dilution).

Measurement of DRA Promoter Activity—Caco-2 cells were transiently transfected with full-length DRA promoter and different deletion constructs cloned upstream of the luciferase reporter gene and *p*-cytomegalovirus (CMV)-*β*, *β*-galactosidase mammalian expression vector (BD Biosciences, Clontech, Palo Alto, CA), by electroporation utilizing a Amaxa nucleofactor system as previously described (33). 24 h post-transfection, cells were treated with ATRA or *RAR* agonist/antagonist for different time periods. Control cells were treated with vehicle (100% alcohol) at 0.1% final concentration. After completion of the treatment, cells were washed with 1× PBS and lysed using passive lysis buffer (Promega, Madison, WI). Activities of luciferase and *β*-galactosidase were measured by a luminometer (Promega), utilizing kits from Promega and Clontech, respectively, according to the manufacturer's instructions. Promoter activity was calculated as a ratio of luciferase value to *β*-galactosidase value for each sample and expressed as % of control. All transfections were performed in triplicate and repeated at least three times with separate batches of cells.

siRNA-mediated Silencing—For small RNA interference studies, Caco-2 cells were plated on 6-well plates at a density of 1×10^5 cells/well, 24 h before transfection. After 24 h, Caco-2 cells were transfected with *RAR-β* (sense: 5′-GCGUGUAAUUACCUUGAAATT-3′, antisense: 5′-UUUCAAGGUAAUUACACGCTC-3′) or *HNF-1β* (sense: 5′-GCUCUGAGCCCACCAACAATT-3′, antisense: 5′-UUGUUGGUGGGCUCAGAGCAG-3′) specific siRNA (100 pmol) and scrambled (control) siRNA (100 pmol) (Qiagen, Valencia, CA) utilizing Lipofectamine 2000 transfection reagent (Invitrogen) as recommended by the manufacturer. Post-siRNA transfection (48 h), cells were treated with ATRA or vehicle for 24 h. Total RNA and protein was extracted as described before. Silencing was validated by real-time PCR utilizing *RAR-β*- or *HNF-1β*-specific primers and measuring the protein expression.

Immunofluorescence Staining and Confocal Microscopy—Caco-2 cells grown on transwell inserts were treated with ATRA for 24 h. After treatment the monolayers were washed twice in 1× PBS containing 1 mM $CaCl_2$, pH 7.4, and then fixed with 2% paraformaldehyde at room temperature. Fixed cells were permeabilized using 0.08% saponin and blocked in 5% normal goat serum for 2 h. Monolayers were then incubated with rabbit anti-human DRA antibody (1:100) for 2 h followed by 3 washes for 5 min with 1× PBS containing $CaCl_2$ and saponin. Cells were finally incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody at 1:100 dilution (Invitrogen) and rhodamine-phalloidin (1:60 dilution; Invitrogen) for 60 min at room temperature. Inserts were carefully removed from the transwell and mounted on glass slides using Slowfade gold antifade reagent (Invitrogen). Images were obtained on Carl Zeiss LSM 510 META laser scanning confocal microscope equipped with a ×63 water-immersion objective. Beams of 488 and 534 nm from an Ag/Kr laser and 361 nm from a UV laser were used for excitation. LP505 and -585 filters were used for detecting green and red fluorescence emissions, respectively. The multitracking function was used to sequentially scan two different fluorochromes to avoid any bleed-through among these fluorescent dyes. A series of sections were taken at *z* direction, and orthogonal sections were made in a *z* stack. The quantitative assessment of the apical amount of DRA was done using actin as an internal marker by Image J software.

Cell Surface Biotinylation Studies—Cell surface biotinylation was performed using sulfo-NHS-SS-biotin (1.5 mg/ml, Thermo Scientific, Rockford, IL) in borate buffer (in mM: 154 NaCl, 7.2 KCl, 1.8 $CaCl_2$, 10 H_3BO_3, pH 9.0) as previously described (24). After ATRA treatment, labeling of cell surface antigens was done for 60 min at 4 °C to prevent endocytosis and internalization of antigens. The biotinylated proteins were extracted from equal amounts of total protein by immunoprecipitation with neutravidin plus ultralink resin. The biotinylated proteins were released by boiling in Laemmli buffer containing dithiothreitol and subjected to SDS-PAGE, followed by transfer to nitrocellulose membrane. The blots were immunostained with anti-DRA antibody. The surface DRA was normalized with total cellular DRA (sum of biotinylated fraction and the amount of DRA not removed by the neutravidin precipitation method (intracellular pool)).

Statistical Analysis—Results are expressed as mean ± S.E. of three to five independent experiments. Student's *t* test or one-way analysis of variance with Tukey's test was used for statistical analysis. Differences between control and treated groups were considered significant at *p* value of 0.05 or less.

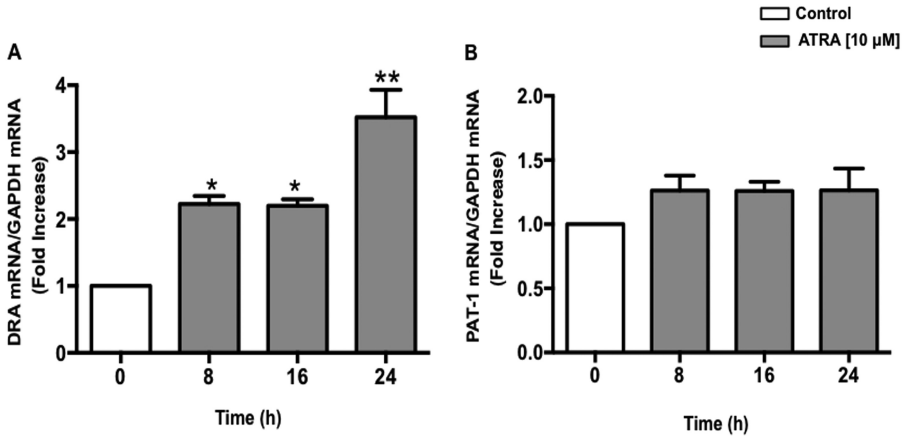

FIGURE 1. **ATRA stimulates DRA but not PAT-1 mRNA levels in Caco-2 cells.** Caco-2 cells were treated with 10 μM ATRA for 8, 16, and 24 h in serum-free cell culture medium. RNA was amplified utilizing DRA (*A*) or PAT-1 (*B*) gene-specific primers for real-time PCR quantification. Data represent the relative expression of DRA or PAT-1 normalized to the respective *GAPDH* mRNA (internal control) levels. Results are expressed as fold-changes in mRNA levels in treated cells as compared with control. Data represent mean ± S.E. of 4 separate experiments. *, $p < 0.05$; **, $p < 0.001$ compared with control.

Results

ATRA Up-regulates DRA but Not PAT-1 mRNA Levels in Caco-2 Cells—Two members of the *SLC26* gene family, DRA (*SLC26A3*) and PAT-1 (*SLC26A6*) have been identified as candidate genes for apical Cl^-/HCO_3^- exchange activity in the mammalian intestine (34). Therefore we examined whether ATRA treatment modulates DRA and/or PAT-1 mRNA expression. Caco-2 cells were treated with 10 μM ATRA for 8, 16, and 24 h and mRNA levels of DRA and PAT-1 were determined. As shown in Fig. 1*A*, mRNA levels of DRA were found to be significantly increased as early as 8 h with ~3.5-fold increase at the 24 h time point. However, PAT-1 mRNA levels remained unaltered in response to ATRA treatment (Fig. 1*B*).

ATRA Increases DRA Protein Expression—Effect of ATRA treatment for 8, 16, and 24 h was next examined on DRA protein expression. Parallel to the effect of ATRA on DRA mRNA, Western blotting data demonstrated a significant increase in DRA protein expression (Fig. 2*A*). Densitometric analysis of the protein bands showed that ATRA treatment increased DRA protein levels by ~50% as compared with control at 24 h. However, no significant change in protein expression was observed at 8- and 16-h time periods (Fig. 2*B*). These results were further supported by our confocal immunofluorescence studies. The vertical XY and horizontal XZ images in Fig. 2*C* show apical localization of DRA (*green*) with actin (*red*) in control cells. ATRA treatment resulted in a significant increase in DRA protein expression (138.3 ± 23.5%) as compared with the vehicle-treated cells taken as 100%. Additionally, cell surface biotinylation studies were utilized to quantify the increase in surface DRA (defined as the biotin accessible fraction of the total cellular DRA level) levels in response to ATRA treatment (Fig. 2*D, i*). Densitometric analysis of the protein bands (surface DRA/total DRA (surface + intracellular) ratio) suggested that ATRA increased the surface DRA level by 43% compared with untreated control (Fig. 2*D, ii*).

ATRA Increases DRA Promoter Activity in Caco-2 Cells—As ATRA increased DRA mRNA levels; we next investigated whether this increase was through a transcriptional mechanism. Caco-2 cells were transiently transfected with DRA promoter construct (p-1183/+114) along with pCMV-β, β-galactosidase expression vector. Post-transfection, cells were treated for 24 h with different concentrations of ATRA (1, 5, and 10 μM), and DRA promoter activity was determined by firefly luciferase assay and the measurement of β-galactosidase as an internal control to correct for transfection efficiency. As shown in Fig. 3*A*, ATRA treatment significantly stimulated DRA promoter activity with 2.5-fold induction at 10 μM concentration. The time course ATRA treatment demonstrated an increase in DRA promoter activity as early as 8 h with a significantly higher increase at the 24 h time point (Fig. 3*B*). Most of the effects of vitamin A are attributable to its active metabolites such as ATRA, 9-*cis*-, and 13-*cis*-retinoic acid. Not only ATRA, but its 9-*cis* and 13-*cis* stereoisomers (10 μM, 24 h) also significantly increased the DRA promoter activity (Fig. 3*C*). These data demonstrate that transcription of DRA is up-regulated by treatment of ATRA and its 9-*cis* and 13-*cis* isomers. However, for all other studies, we used ATRA, as it is the predominant and most active form under most physiological situations (35).

Effect of ATRA on DRA Promoter Deletion Constructs—We next aimed to identify the ATRA responsive region mediating the stimulatory effect of ATRA on DRA promoter activity. Caco-2 cells were transiently transfected with various constructs representing progressive 5′ deletions in the DRA promoter. Cells were then treated with ATRA (10 μM) for 24 h, and promoter activity was measured by luciferase assay. As shown in Fig. 4, ATRA treatment resulted in a significant increase in the relative luciferase activity of each deletion construct compared with their respective controls taken as 100%. The stimulatory effect of ATRA was retained until the smallest construct flanking the region between −179 and +114 of the DRA promoter was reached. These results indicate that ATRA-mediated activation required only the shortest fragment of the DRA promoter and ATRA response elements are located in this region of DRA gene.

RAR-β Receptor Is Involved in the Induction of DRA Promoter Activity—Effects of ATRA are receptor mediated. It is known that ATRA regulates the expression of its own receptors. Keeping this in view, we first examined the expression of *RAR* iso-

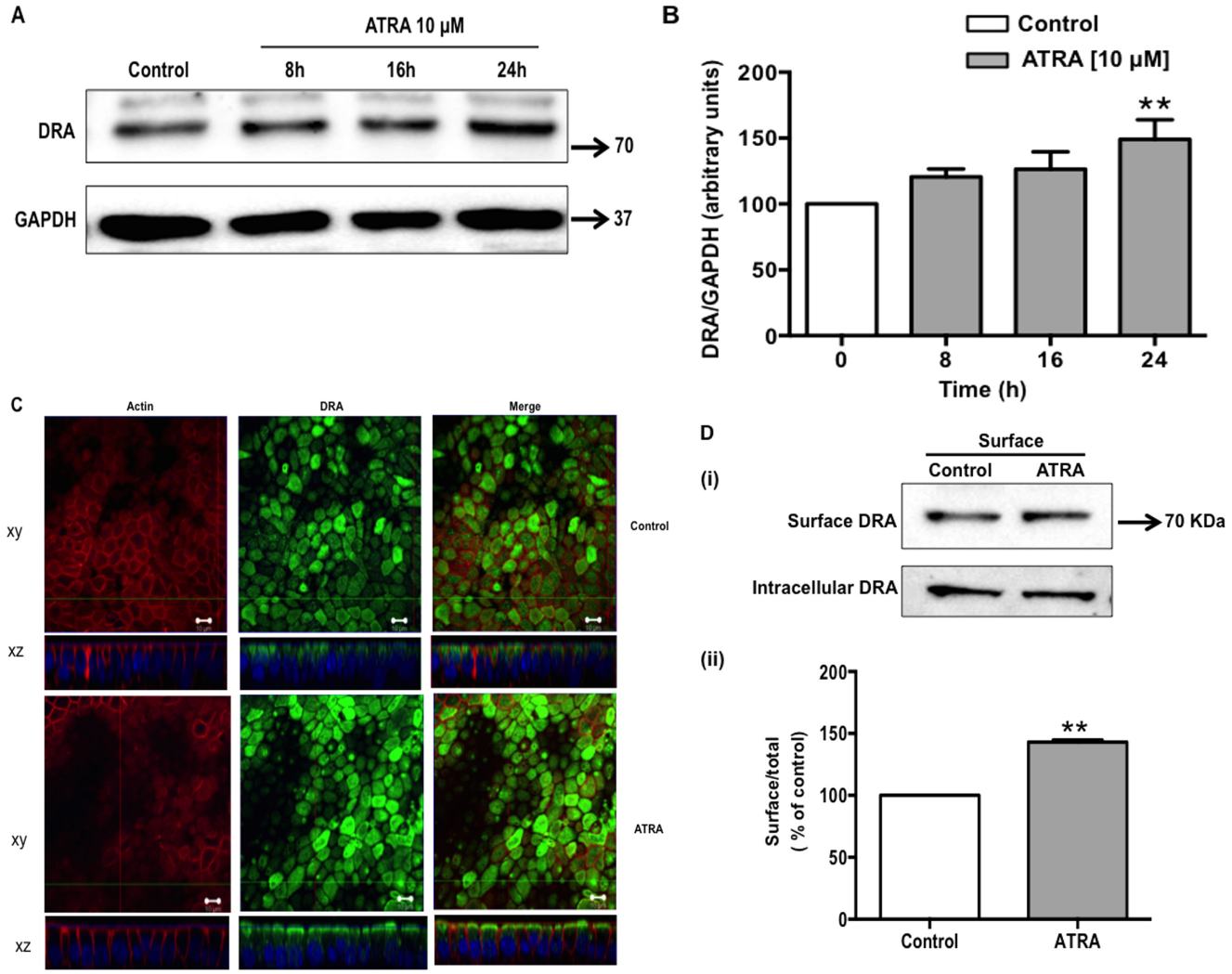

FIGURE 2. **ATRA induces DRA protein expression.** Caco-2 cells were treated with 10 μM ATRA for 8, 16, and 24 h in serum-free cell culture medium. Control and ATRA-treated cell lysates were subjected to 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. *A,* the blot was probed with rabbit anti-DRA or anti-*GAPDH* antibody. A representative blot of 3 separate experiments is shown. *B,* results of densitometric analysis are expressed as DRA/*GAPDH* levels. *C,* confocal microscopic localization of DRA (*green*) and Alexa Fluor 568-conjugated phalloidin (actin; *red*) and slow fade gold antifade reagent with DAPI labeled the nuclei (*blue*). ATRA (10 μM for 24 h) treated cells show an enhanced expression of the DRA on the apical surface compared with control. Representative image from 3 separate experiments is shown. *D,* Caco-2 monolayers were treated with ATRA (10 μM) in serum-free cell culture medium for 24 h and subjected to biotinylation at 4 °C using sulfo-NHS-SS-biotin. Surface and intracellular fractions were run on 7.5% SDS-PAGE followed by transfer to nitrocellulose membrane. The blot was immunostained with a rabbit anti-DRA antibody. (*i*) Representative blot of 3 different experiments is shown. (*ii*) Data were quantified by densitometric analysis and expressed as surface/total DRA (surface plus intracellular DRA). Values represent mean ± S.E. of 3 different experiments. **, $p < 0.001$ compared with control.

forms in response to ATRA treatment. As shown in Fig. 5*A*, ATRA treatment resulted in a 12-fold increase in *RAR-β* mRNA expression as compared with control, with a modest change in *RAR-α* or *-γ*. Suggesting that RAR-*β* might play a role in the observed effects of ATRA. To confirm the involvement of *RAR-β*, Caco-2 cells transiently transfected with the DRA promoter were treated with the *RAR-β* agonist (CH-55, 1 μM) alone or in combination with ATRA for 24 h. As shown in Fig. 5*B*, treatment with the *RAR-β* agonist significantly increased the DRA promoter activity similar to the effect of ATRA. However, the combined effects of ATRA and CH-55 on DRA promoter activity were not additive indicating that both ATRA and CH-55 increase DRA promoter activity by the same pathway via *RAR-β*. The role of *RAR-β* was further confirmed by treatment of the DRA promoter-transfected Caco-2 cells with *RAR-β* antagonist (LE-135). As shown in Fig. 5*C*, an ATRA-mediated increase in DRA promoter activity was completely abrogated in the presence of LE-135 (1 μM). These results indicate that *RAR-β* is involved in stimulating DRA promoter activity. Effects of *RAR-β* agonist and antagonist observed on the DRA promoter were further validated at the protein level. Parallel to ATRA, CH-55 treatment of Caco-2 cells for 24 h also resulted in a significant increase (~50%) in DRA protein expression. Whereas, the ATRA-induced increase in DRA protein levels were abrogated in the presence of LE-135 (Fig. 5*D*, *i* and *ii*).

As a complementary approach, we also examined the effect of *RAR-β* knockdown on ATRA-induced DRA expression. Treatment of Caco-2 cells with *RAR-β*-specific siRNA duplex for 48 h significantly decreased the *RAR-β* mRNA expression (~70% decrease) compared with scrambled siRNA controls, demonstrating efficient knockdown. This was also validated at

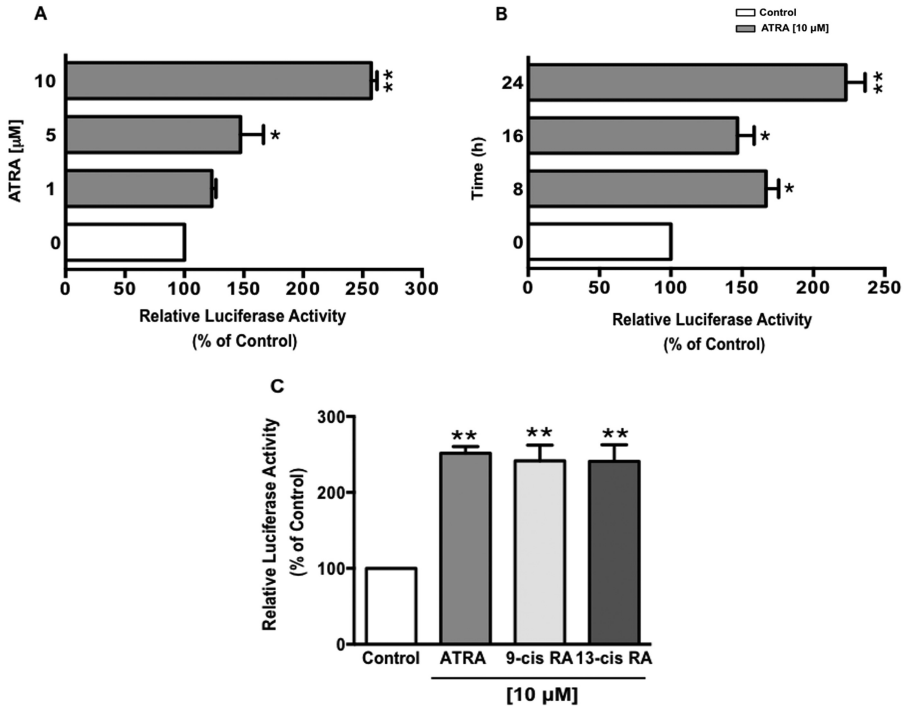

FIGURE 3. **ATRA stimulates DRA promoter activity in a dose-dependent manner.** Caco-2 cells were transiently transfected with DRA luciferase promoter construct (p-1183/+114) along with the mammalian expression vector for β-galactosidase (pCMVβgal). 24 h post-transfection, Caco-2 cells were treated with: *A,* different doses of ATRA ranging from 1 to 10 μM for 24 h; or *B,* 10 μM ATRA for different time points; or *C,* 10 μM, ATRA, 9-*cis*- or 13-*cis*-retinoic acid. Promoter activity was measured by luciferase assay. Values were normalized to β-galactosidase activity to correct for transfection efficiency. Results represent mean ± S.E. of 4 separate experiments performed in triplicate and expressed as % of control comparing transfected cells treated with ATRA to vehicle-treated cells (control). *, $p < 0.05$; **, $p < 0.001$ compared with control.

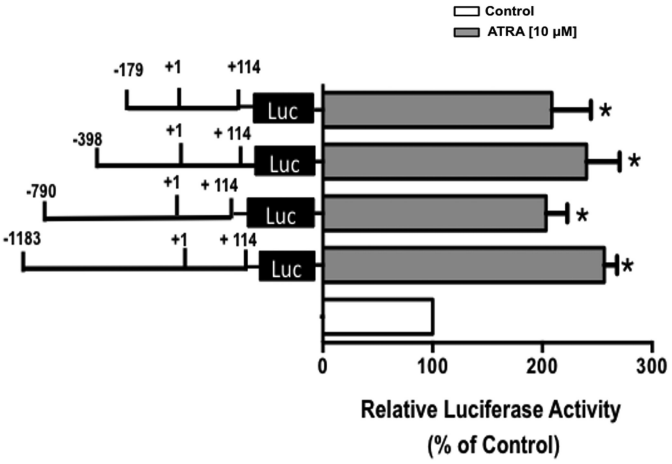

FIGURE 4. **Functional analysis of DRA promoter deletion constructs in response to ATRA treatment.** Caco-2 cells were transiently transfected with full-length DRA promoter (p-1183/+114) and its progressive 5′ deletion constructs along with pCMVβgal vector. At 24 h post-transfection, cells were treated with 10 μM ATRA for 24 h. Cells were then harvested 48 h post-transfection and the promoter activity was measured by luciferase assay. Values were normalized to β-galactosidase activity to correct for transfection efficiency. Results are expressed as % of respective control for each promoter construct and represent mean ± S.E. of 3 separate experiments performed in triplicate. *, $p < 0.05$ compared with respective control taken as 100%.

the protein level (Fig. 5*E, i*). As shown in Fig. 5*E*, *ii*, ATRA treatment to cells transfected with scrambled siRNA (control) resulted in a significant increase in DRA mRNA expression. However, similar to the inhibition by LE-135 (*RAR-β* antagonist), transfection of *RAR-β*-specific siRNA substantially attenuated the ability of ATRA to increase DRA expression. These findings clearly indicate the involvement of *RAR-β* in the ATRA-induced increase in DRA expression.

HNF-1β Mediates the Effect of ATRA on DRA—Results obtained from the transfection studies with progressive 5′-deletion constructs of the DRA promoter demonstrated that the minimal promoter region has *cis*-elements mediating the ATRA-induced effects on DRA promoter. Interestingly, the sequence analysis of this region of the DRA promoter revealed the binding sites for hepatocyte nuclear factors (*HNF*s). In this regard a previous study has suggested transcriptional regulation of DRA expression by *HNF*s (36). Complete loss of DRA expression has been shown in the small intestine and colon of intestine-specific *HNF-1α* and *-1β* double knock-out mice (29). Thus the potential involvement of HNFs in the ATRA-induced increase in DRA expression was evaluated. Caco-2 monolayers were treated with ATRA (10 μM) for 24 h and the expression levels of *HNF*s (*1α*/*1β*/*4α*) were examined. ATRA markedly increased *HNF*-1β mRNA expression as compared with *HNF-1α* and *HNF*-4α (Fig. 6*A*). The time-dependent increase in *HNF-1β* mRNA expression in response to ATRA (Fig. 6*B*) showed a similar pattern as observed for DRA mRNA. These data suggest that *HNF-1β* may be involved in mediating the effects of ATRA on DRA. To confirm this, *HNF-1β* expression was attenuated in Caco-2 cells using siRNA. Treatment of Caco-2 cells with the *HNF-1β*-specific siRNA duplex for 48 h significantly decreased the *HNF-1β* mRNA expression (~50% decrease) compared with scrambled siRNA controls, demonstrating efficient knockdown. This was also validated at the protein level (Fig. 6*C, i*). As shown in Fig. 6*C, ii*, ATRA significantly

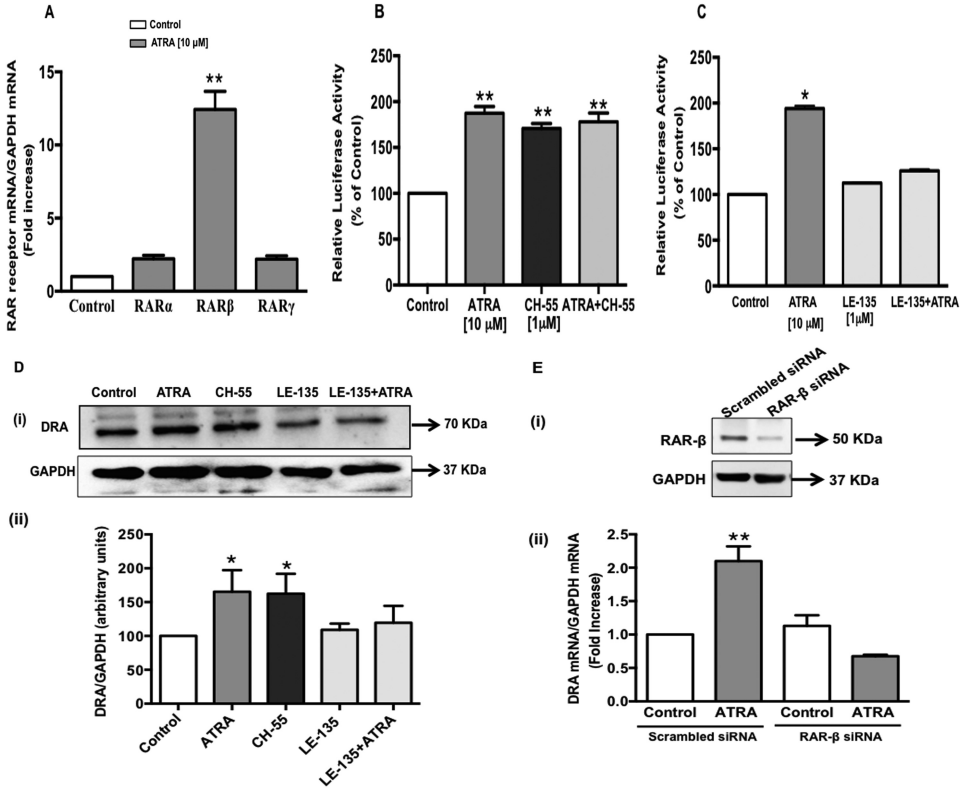

FIGURE 5. ***RAR-β* receptor is involved in the induction of DRA promoter activity.** *A*, Caco-2 cells were treated with 10 μM ATRA for 24 h in serum-free cell culture medium. RNA was amplified utilizing *RAR-α*, *-β*, or *-γ* gene-specific primers for real-time PCR quantification. Data represent the relative expression of *RAR-α*, *-β*, or *-γ* normalized to the respective *GAPDH* mRNA (internal control) levels and are expressed as fold-changes compared with vehicle-treated controls. Values represent mean ± S.E. of 3 separate experiments performed. **, $p < 0.001$ compared with respective control. *B* and *C,* Caco-2 cells were transiently transfected with DRA luciferase promoter construct (p-1183/+114) along with pCMVβgal vector. *B*, after 24 h, cells were treated with *RAR-β* receptor agonist CH-55 (1 μM) or ATRA (10 μM) or both for 24 h. *C,* transiently transfected cells were pretreated with *RAR-β* receptor antagonist LE-135 (1 μM) for 60 min and then coincubated with ATRA (10 μM) for 24 h. Promoter activity was measured by luciferase assay. Values were normalized to β-galactosidase activity to correct for transfection efficiency. Results are expressed as % of control and represent mean ± S.E. of 3 separate experiments. *, $p < 0.05$; **, $p < 0.001$ compared with control. *D,* Caco-2 cells were treated with ATRA (10 μM) or CH-55 (1 μM) or LE-135 (1 μM) alone or in combination with ATRA for 24 h in serum-free media. Cell lysates prepared from the above treatment groups were subjected to 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. (*i*) The blot was probed with rabbit anti-DRA or anti-*GAPDH* antibody. A representative blot of 3 separate experiments is shown. (*ii*) Results of densitometric analysis are expressed as DRA/*GAPDH* levels. Values represent mean ± S.E. of 3 different experiments. *, $p < 0.05$ compared with control. *E,* Caco-2 cells were transfected with scrambled or *RAR-β*-specific small interfering RNA (siRNA) for 48 h. (*i*) siRNA-mediated knockdown of *RAR-β* was confirmed by immunoblots with anti-*RAR-β* antibody. (*ii*) in a separate set of experiments, the cells were treated with 10 μM ATRA for an additional 24 h. Total RNA was extracted and quantitative real-time RT-PCR was performed utilizing primers specific for DRA. Data represent the relative expression of DRA normalized to the respective *GAPDH* mRNA (internal control) levels. Results are expressed as fold-changes in mRNA levels compared with control. Values represent mean ± S.E. of 3 separate experiments. **, $p < 0.001$ compared with control.

increased DRA mRNA levels in the cells transfected with scrambled siRNA. siRNA-mediated silencing of *HNF-1β* resulted in ~35% decrease in basal DRA mRNA expression. However, the ATRA-induced increase in DRA mRNA levels was significantly decreased by *HNF-1β* knockdown. These data suggest that ATRA modulates DRA expression by increasing *HNF-1β* in intestinal Caco-2 cells.

RAR-β Is Upstream of HNF-1β—As the siRNA-mediated silencing of both *RAR-β* and *HNF-1β* blocked the effects of ATRA on DRA mRNA expression; we next sought to determine the sequence of events involved. As shown in Fig. 7*A*, *i* and *ii*, ATRA treatment to Caco-2 cells transfected with scrambled siRNA resulted in a significant increase in *HNF-1β* mRNA and protein levels and this increase was abolished by *RAR-β* knockdown. However, the levels of *RAR-β* mRNA and protein, in response to ATRA treatment remained elevated with *HNF-1β* silencing (Fig. 7*B*, *i* and *ii*) indicating that in the sequence of events *RAR-β* is upstream of *HNF-1β*.

Discussion

DRA functions as the key mediator of apical Cl^-/HCO_3^- exchange in the electroneutral NaCl absorption process in the intestinal epithelial cells. Disturbances in NaCl absorption accompany diarrhea associated with inflammatory bowel diseases and pathogenic bacterial infections (23). Therefore, the maintenance of optimal expression of DRA is critical for normal electrolyte homeostasis in the intestine and mechanisms regulating DRA expression assume added importance in understanding the pathophysiology of diarrheal diseases. Results from the current study demonstrate that ATRA increases DRA gene expression and promoter activity in intestinal epithelial cells. Furthermore, ATRA-mediated stimulation of DRA promoter through *RAR-β* appears to be indirect, involving the increased gene expression of another transcription factor *HNF-1β*. This novel mechanism of up-regulating DRA expression in intestinal epithelial cells may have therapeutic importance in the treatment of diarrheal diseases.

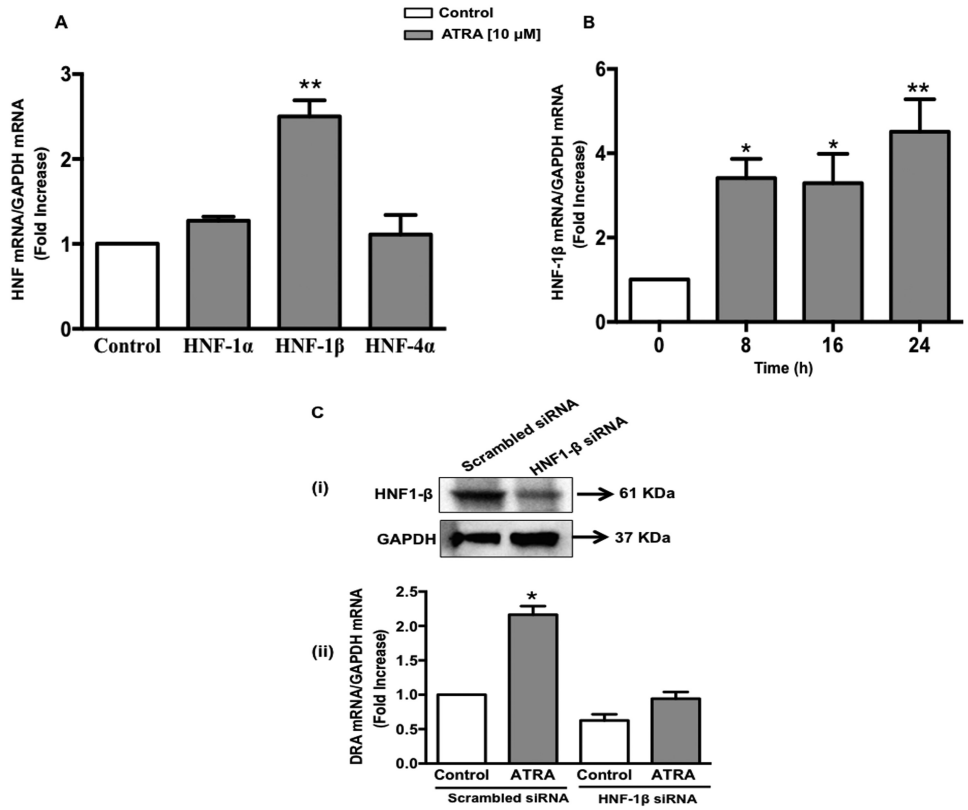

FIGURE 6. **ATRA-mediated stimulation of DRA promoter activity is *HNF-1β* dependent.** *A,* Caco-2 cells were treated with 10 μM ATRA for 24 h in serum-free cell culture medium. RNA was amplified utilizing *HNF-1α*, *-1β*, or *-4α* gene-specific primers. Data represent the relative expression of *HNF-1α*, *-1β*, or *-4α* normalized to the respective GAPDH mRNA (internal control) levels. *B,* Caco-2 cells were treated with 10 μM ATRA for 8, 16, and 24 h in serum-free cell culture medium and *HNF-1β* mRNA expression was determined using gene-specific primers. Results are expressed as fold-change in mRNA levels in ATRA treated as compared with vehicle-treated control cells ($n = 3$, *, $p < 0.05$; **, $p < 0.001$); *C,* Caco-2 cells were transfected with scrambled or *HNF-1β*-specific small interfering RNA (siRNA) for 48 h. (*i*) siRNA-mediated knockdown of *HNF-1β* was confirmed by immunoblotting with anti-HNF1-*β* antibody. (*ii*) In a separate set of experiments, the cells were treated with 10 μM ATRA for an additional 24 h. Total RNA was extracted and quantitative real-time RT-PCR was performed utilizing DRA gene-specific primers. Data represent the relative expression of DRA normalized to the respective *GAPDH* mRNA (internal control) levels. Results are expressed as fold-change in mRNA levels of treated as compared with control cells. Data represent mean ± S.E. of 3 separate experiments. *, $p < 0.05$ compared with control.

ATRA is required for growth, maintenance, and differentiation of intestinal epithelial cells. Notably, the epithelial cells lining the gastrointestinal tract are exposed to varying concentrations of this dietary nutrient (37, 38). In addition, the absorbed dietary retinol is metabolized directly to ATRA in the enterocytes (1), and ATRA is the primary retinoid that affects the cellular process at the level of transcription. Our results showed that the promoter activity of DRA is significantly increased parallel to an increase in the mRNA expression of DRA by ATRA, suggesting the involvement of transcriptional regulation. The effects on DRA were specific as PAT-1 mRNA remained unchanged in response to ATRA treatment. DRA coexists with NHE-3 in distal ileum and mid-colon, and the coupled operation favors electroneutral NaCl absorption. The expression profiles of both DRA and NHE-3 exhibit regional variations along the length of the intestine as well as vertically along the crypt-villus axis (39, 40). NHE-3 expression is more in ileum as compared with colon and the pattern is inverse for DRA. An increase in expression of both DRA and NHE-3 has been reported by lysophosphatidic acid (27, 28, 41) and the probiotic *L. acidophilus* (25, 26, 42). Thus, in future studies it will be interesting to examine the changes in the expression pattern of NHE-3 and DRA *in vivo* in response to ATRA treatment.

ATRA signaling is mediated by its binding to *RAR*s, which form heterodimers with *RXR*s. It is established that ATRA exerts its effects by differentially regulating gene expression of its receptor isotypes such as *RAR-α* and *RAR-β* (43), which also determines the sensitivity of the individual cell lines to the effect of ATRA (44, 45). The present study utilized the well established human intestinal epithelial cell line Caco-2 as an *in vitro* model to understand mechanisms underlying ATRA-mediated effects on DRA. Caco-2 cells represented the excellent *in vitro* model for our studies, as on differentiation these cells manifest many anatomic and functional similarities to absorptive enterocytes. Previous studies have demonstrated that retinoic acid receptors are expressed in Caco-2 cells (1, 37, 38). In our study, ATRA treatment of Caco-2 cells caused a highly significant (~12-fold) increase in *RAR-β* mRNA. In this regard, previous studies in hepatic epithelial cells have also shown a similar up-regulation of *RAR-β* in response to ATRA, mediated by binding the *RXR*/*RAR* heterodimer to the promoter region of the *RAR-β* gene (43). A question arises whether *RXR* is also involved in the observed effects of ATRA on DRA. However, in the current study, DRA promoter activity and RNA expression both remained unaffected in the presence of *RXR* agonist (DHA) and antagonist (HX531) (data not shown) suggesting that transcriptional activation of the DRA promoter depends

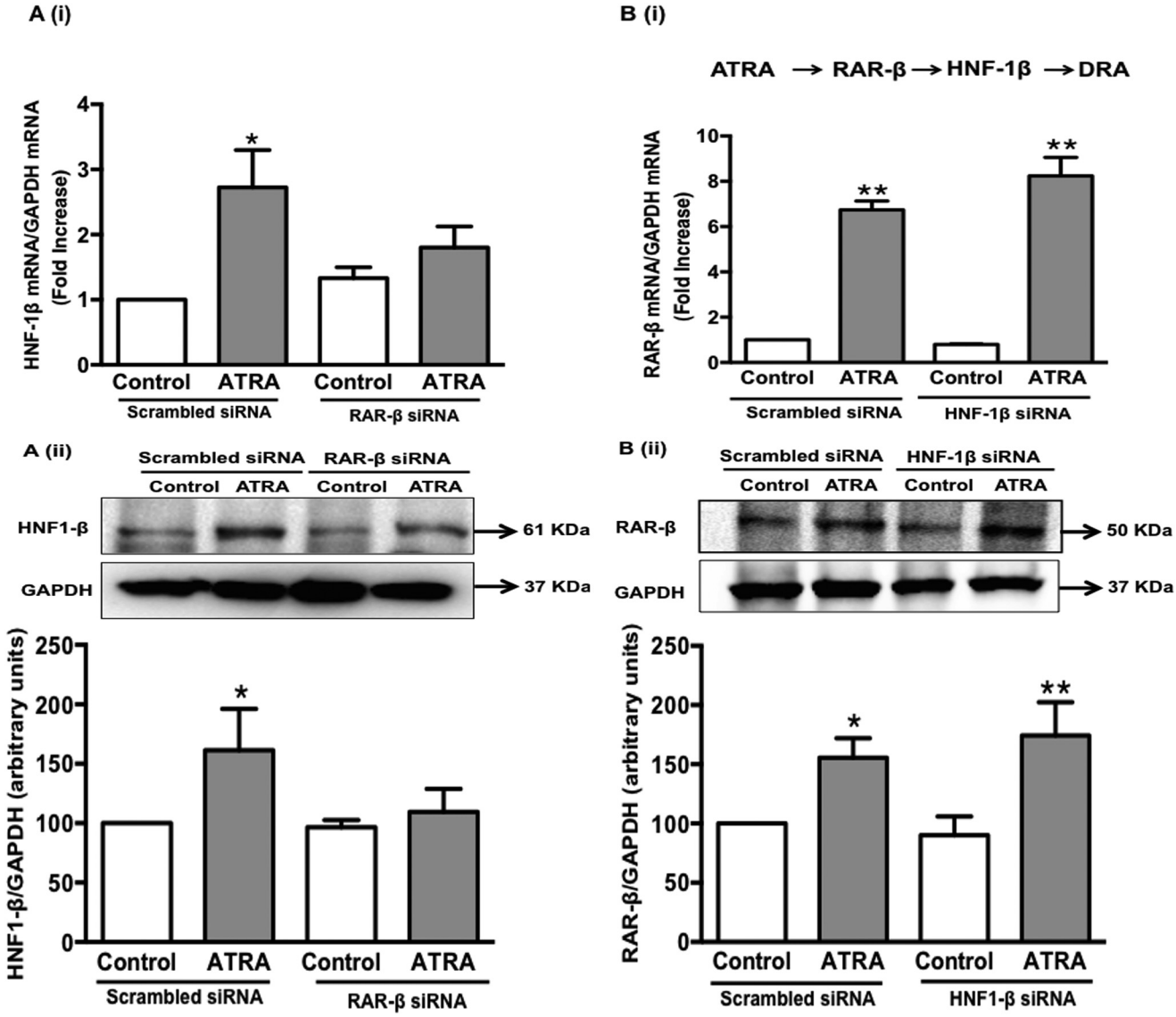

FIGURE 7. ***RAR-β* is upstream of *HNF-1β* in ATRA-mediated stimulation of DRA expression.** Caco-2 cells were transfected with scrambled, HNF-1β-specific and *RAR-β*-specific small interfering RNA (siRNA) for 48 h. The cells were then treated with 10 μM ATRA for an additional 24 h. Total RNA and protein was extracted. *A*, (*i*) quantitative real-time RT-PCR was performed utilizing *HNF-1β* gene specific primers and (*ii*) protein expression of *HNF-1β* was examined using anti-HNF1-β antibody in cells where *RAR-β* was silenced. *B*, (*i*) quantitative real-time RT-PCR was performed utilizing *RAR-β* gene-specific primers and (*ii*) protein expression of *RAR-β* was examined using anti-RAR-β antibody in the cells where *HNF-1β* was silenced. Data represent the relative expression of *HNF-1β* or *RAR-β* normalized to the respective *GAPDH* mRNA (internal control) levels. Results are expressed as fold-changes in mRNA levels compared with control. For protein expression, a representative blot of 3 separate experiments is shown. Results of densitometric analysis are expressed as *HNF-1β* or RAR-β/*GAPDH* levels. Values represent mean ± S.E. of 3 different experiments. *, $p < 0.05$; **, $p < 0.001$ compared with control.

on binding of ATRA to the RAR partner of *RAR*/*RXR* heterodimer. Interestingly, our results show that DRA promoter activity was stimulated by pharmacological activation of *RAR-β* and the effects were blocked in the presence of *RAR-β* antagonist (LE-135). Also, the increase in DRA mRNA expression by ATRA was abrogated by *RAR-β* knockdown in Caco-2 cells. Collectively, these results suggest that ATRA effects on DRA gene expression are dependent on *RAR-β*. Our studies, however, do not rule out the involvement of RAR-α in the ATRA-mediated effects on DRA. It is important to note that *RAR-α* has been previously identified as a critical activator of ATRA-mediated RAR-β gene expression in human mammary epithelial cells (46) and cervical cells (47). A similar involvement of *RAR-α* can be expected in the ATRA-induced increase in DRA gene expression via *RAR-β*, and future investigations will address this possibility.

ATRA and its derivatives are dietary factors, which regulate cellular differentiation. As expression of DRA increases with enterocyte maturation, it was of interest to examine whether the ATRA-induced DRA expression is through the regulation of DRA promoter activity or is secondary to alterations in the cellular differentiation/proliferation status of the Caco-2 cells. To test this, the effect of ATRA on alkaline phosphatase and proliferating cell nuclear antigen, markers of differentiation and proliferation, respectively, was assessed. ATRA (10 μM) treatment to pre-confluent (24 h post-plating) and post-confluent (14 days post-plating) Caco-2 cells for 24 h had no significant effect on alkaline phosphatase and proliferating cell

nuclear antigen expression (data not shown). This indicates that it is unlikely that the observed increase in DRA expression is secondary to cellular differentiation.

Basic sequence pivotal for the classical ATRA signaling pathway involves ligand binding, receptor dimerization, DNA binding, and transcriptional modulation of the target gene. However, there are possibilities where ATRA regulates an intermediary factor (usually another transcription factor), which in turn regulates the target gene (indirect target) (48). Sequence analysis of the DRA promoter revealed the potential binding sites for *RAR-β*. However, it should be noted that deletion of DRA promoter regions harboring the *RAR-β* binding sites retained significant responsiveness to ATRA. These data indicated that other potential *cis*-elements present in the p-179/+114 region of DRA promoter might have contributed to ATRA-mediated stimulation of DRA promoter activity. Sequence analysis of this region revealed a potential *HNF-1β* recognition site located within the −94/−76 region from the transcription start site ($^{-94}$AGTTAATGAGAGTTAATTA^{-76}). ATRA treatment resulted in a significant increase in *HNF-1β* mRNA expression (as compared with *HNF-1α* and *-4α*), which was abrogated by *RAR-β* knockdown in Caco-2 cells. On the other hand, *HNF-1β* silencing resulted in blocking the stimulatory effects of ATRA on DRA mRNA expression, whereas *RAR-β* mRNA and protein levels remained up-regulated. These data indicated that the effects of ATRA on DRA expression are mediated by transcriptional up-regulation of *HNF-1β*. In this regard, indirect regulation of α-fetoprotein by ATRA via *HNF-1α* and *HNF-4α* in the hepatic cell line has also been reported earlier (49). In the intestine, *HNF*s play a key role in regulating the expression of genes involved in diverse physiological processes such as those related to differentiation, cell fate, barrier function, transport, and metabolism (29). With respect to the regulation of transporter genes, *HNF-1α* has been shown to regulate *CFTR* gene expression in Caco-2 cells (50). On the other hand, a recent study has shown that the expression of ileal and colonic DRA were substantially reduced in mice deficient in either intestinal *HNF-1α* or *HNF-1β*, and were completely abolished in mice deficient in both factors in the intestine (29). These mice also exhibited diarrheal phenotype. Additionally, our previous report on regulation of the basal DRA promoter by *HNF-4α* (36) suggest that HNFs might play critical roles in modulating DRA expression in the normal intestine and its alterations in inflammation. Our current findings further indicate a novel role for *HNF-1β* in mediating the effects of ATRA on DRA expression. It is intriguing that DRA mRNA and promoter activity showed an equal amount of increase at 8 and 16 h, which further increased at the 24-h time point. The time-dependent increase in *HNF-1β* expression in response to ATRA also showed a similar pattern as observed for DRA mRNA. We speculate that the ATRA-induced increase in *HNF-1β* mRNA expression may reflect the involvement of two mechanisms including an increase in *HNF-1β* promoter activity maximally as early as 8 h and a secondary mechanism involving chromatin remodeling at later time points in response to ATRA treatment, which is subsequently reflected in the expression pattern of the target gene (DRA). In this regard, a recent study has shown that ATRA can induce chromatin remodeling (51).

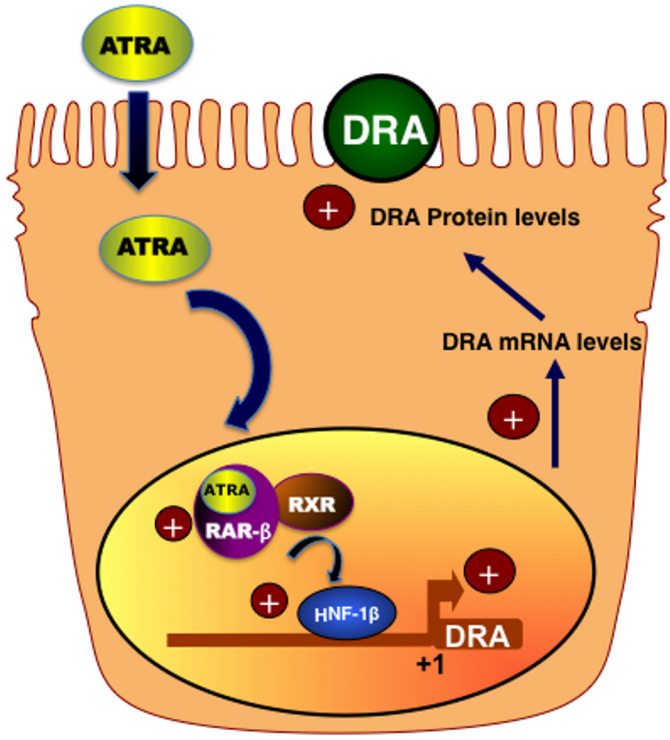

FIGURE 8. **Proposed model for the effects of ATRA on DRA via *HNF-1β*.**

In conclusion, our present studies provide novel data on the up-regulation of DRA expression by ATRA via transcriptional mechanisms. We propose a model (Fig. 8) that ATRA through *RAR-β* stimulates the promoter activity of DRA via *HNF-1β* leading to induction of transcription and subsequent increase in DRA message and protein expression. This study highlights mechanisms that underlie potential antidiarrheal effects of ATRA. These findings are clinically relevant, as induction of DRA expression may be beneficial in conditions like diarrhea associated with inflammatory bowel diseases where DRA expression is down-regulated. However, future studies are needed to verify whether ATRA has an impact on DRA gene expression *in vivo* in normal and disease models.

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