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Suppression of Hepatitis B Virus Core Promoter by the Nuclear Orphan Receptor TR4*

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The TR4 orphan receptor is a member of the nuclear receptor superfamily that modulates gene expression via binding to the AGGTCA direct repeat hormone response element. Here we report a functional study of TR4 interaction with the core promoter of the hepatitis B virus (HBV). The electrophoretic mobility shift assay shows that TR4 can bind to the direct repeat 1 sequence element (AGGTTAAAGGTCT, nucleotide coordinates 1757–1769, TR4RE-HBV) on the HBV core promoter. TR4 also can enhance the activity of a synthetic luciferase reporter linked with four copies of TR4RE-HBV in either liver HepG2 or non-liver H1299 cells in a dose-dependent manner. Surprisingly, TR4 represses the activity of a luciferase reporter containing the entire HBV genome sequences. Moreover, mutation of this TR4RE-HBV site in the HBV core promoter diminishes the TR4 suppression effect. This TR4-induced suppression of HBV core promoter activity is further confirmed by primer extension analysis of the HBV core RNAs, showing that TR4 represses both pre-core and core mRNAs. Further dissection of this repressive mechanism indicates that TR4 may suppress the HBV core promoter activity via repressing HNF4α-mediated transactivation by protein-protein interactions without inhibition of HNF4α DNA binding. Furthermore, our results indicate that the N- and C-terminal regions of TR4 protein are required for TR4-HNF4α interaction. It is possible that TR4-HNF4α interaction may block the HNF4α function that results in the suppression of HBV gene expression. Together, these results demonstrate that TR4 can serve as a negative modulator in the transcriptional regulation of HBV core gene expression.

Hepatitis B virus (HBV)[1] is a small virus with a 3.2-kb partially double-stranded DNA genome, containing four open reading frames: the surface antigen, the core antigen, the polymerase, and the X protein (1, 2). HBV is a major pathogen causing acute and chronic liver diseases, such as cirrhosis and hepatocellular carcinoma (3, 4). HBV transcripts are regulated by the transcriptional control of four different promoters and two enhancer elements (5). HBV is replicated from a RNA intermediate as a template for reverse transcription (1). The core promoter controls the expression of the 3.5-kb core mRNA (C mRNA) as the template for replication, and the expression of the pre-core mRNA (pre-C mRNA) for the translation of the HBeAg precursor (6). Recently, multiple binding sites for liver-specific or ubiquitous transcription factors and several hormone response elements (HRE) have been identified in the core promoter region, including Sp1 (7, 8), TATA-binding protein (9), HNF3 (10), HNF4α (11), C/EBP (12), and other members of the nuclear receptor superfamily, such as the retinoid X receptor (RXRα), peroxisome proliferator-activated receptor (PPARα), COUP-TF1, and ARP1 (13). For example, HNF4α and RXRα/PPARα can stimulate the expression of the 3.5-kb core RNA, COUP-TF1 suppresses the expression of both the pre-C RNA and C RNA, and TR2 preferentially represses the expression of the pre-C RNA (14). These findings suggest that these nuclear receptors may play important roles in viral transcription. The detailed mechanisms of how the core promoter is differentially regulated by these nuclear receptors remain unclear.

The testicular orphan receptor 4 (TR4) (NR2C2) is an orphan member of the nuclear receptor superfamily (15–17). This family includes classic steroid hormone receptors, the thyroid hormone receptor, the retinoid receptors, and many nuclear orphan receptors whose physiological ligands have not been identified (18). Members of this superfamily typically contain two major conserved domains, the DNA-binding domain (DBD) and the ligand-binding domain (LBD) (18). Most nuclear receptors bind to their cognate ligands, translocate into the nucleus, and bind to the specific HREs to turn on their downstream target genes. TR4 was isolated from human prostate and testis cDNA libraries and is considered to be an orphan receptor, because its ligand has not been found (15). Our previous data showed that TR4 recognizes different AGGTCA direct repeat (DR) sequences either inducing or suppressing its target genes. For example, TR4 induces reporters containing the DR1 site of human ciliary neurotrophic factor α receptor promoter and the DR4 site of the α-myosin heavy chain promoter (19, 20). In contrast, TR4 suppresses RXRα/retinoic acid receptor (DR1), vitamin D3 receptor (DR3), and RXRα/PPARα (DR1)-mediated

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[1] The abbreviations used are: HBV, hepatitis B virus; TR4, testicular receptor 4; HNF4α, hepatocyte nuclear factor 4 α; DR, direct repeat; RXR, retinoid X receptor; PPARα, peroxisome proliferator-activated receptor α; TR2, testicular receptor 2; C mRNA, core mRNA; pre-C mRNA, pre-core mRNA; C, core; HRE, hormone response element; COUP-TF1, chicken ovalbumin upstream promoter-transcription factor 1; VDR, vitamin D receptor; DBD, DNA-binding domain; LBD, ligand-binding domain; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase.

transactivation as shown by the reporter assay (21–23). These results suggest that TR4 may act as a negative regulator of its target genes containing hormone response elements (HREs) by competition with other nuclear receptors for binding to the HREs (21, 23). In addition, our previous data showed that TR4 could also suppress androgen receptor-mediated transactivation via protein-protein interaction (22).

The distribution of TR4 expression is ubiquitous in many tissues, including the central nervous system, adrenal gland, spleen, thyroid gland, and liver (16, 19, 23). Also, there are many other nuclear receptors expressed in the liver, such as HNF4α (24) and RXRα/PPARα (25, 26). These findings suggest that these nuclear receptors in the liver can coordinately regulate gene expression by interacting with HRE sequences in the promoters of their target genes. Our data show that TR4 can interfere with a number of other nuclear receptors by competing for binding to the same HREs (21, 22).

In this report, we have analyzed the DR1 site in the HBV core promoter. We tested whether the TR4 plays a role in regulating the activity of the core promoter through binding to the HRE-DR1 site. We showed that TR4 binds to the DR1 site in the HBV core promoter and strongly activates a reporter containing synthetic DR1 response elements. Moreover, we demonstrated that TR4 could suppress both pre-C and C RNA expression, which was also confirmed by the luciferase assay using a reporter containing the entire core promoter. Our data further suggest that TR4 may suppress HBV core promoter activity via the interruption of HNF4α-induced HBV core promoter by protein-protein interaction with HNF4α. These data demonstrate that TR4 can function as a repressor in liver cells to suppress HBV gene expression and replication.

MATERIALS AND METHODS

Plasmids—The plasmids CpFL(4)LUC, CpLUC, XpHNF4LUC, and CpM2LUC were gifts from Dr. A. McLachlan (The Scripps Research Institute) and were described previously (13). The plasmid pCDNAI-HNF4α was a gift from Dr. M. Hadzopoulou-Cladaras (Boston University, Boston, MA). pG4LUC was a gift from Dr. K. L. Guan (University of Michigan Medical School, Ann Arbor, MI). The pCMX-TR4, pET14b-TR4, pSG5-VDR, and pCMV-TR2 plasmids were constructed as described previously (27, 28). pWTDHBV contains the head-to-tail dimer of the wild type HBV genome DNA (*adw2*) (8). The GST-TR4, TR4-N-DBD, and TR4-LBD fusion constructs were described in our previous study (29). The GAL4-HNF4α expression plasmid for one-hybrid assay was made by inserting the PCR-generated *Bam*HI/*Xba*I fragment (amino acids 1–465) of HNF4α from pCDNAI-HNF4α into pM containing the GAL4 DBD-(1–147) (Invitrogen).

Electrophoretic Mobility Shift Assay—The EMSA reaction was performed as described previously (20). Briefly, TR4 and HNF4α proteins for EMSA were transcribed and translated in TNT reticulocyte lysates (Promega), according to the manufacturer's instructions. The oligonucleotide probes for EMSA were end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (New England BioLabs). HepG2 cell nuclear extracts were prepared according to the procedure of Andrew and Faller (30). The EMSA reactions were performed with 10 μg of nuclear extracts or 2 μl of TNT lysates in an EMSA buffer containing 10 mM Hepes (pH 7.9), 6% (v/v) glycerol, 2% (v/v) Ficoll, 100 mM KCl, 0.5 mM EDTA, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol. The reaction mixtures of the proteins and DNA were incubated for 15 min at room temperature. For the antibody supershift assay, the mixtures were incubated for another 15 min in the presence or absence of mouse anti-TR4 monoclonal antibody (#15). The protein·DNA complexes were analyzed on a 5% native polyacrylamide gel.

Cell Culture, Transfections, and Luciferase Assays—HepG2 cells, Huh7 (human differentiated hepatoma cells), H1299 cells, or HepG2.1 cells (human de-differentiated hepatoma cells) were maintained in Dulbecco's modified Eagle's medium or RPMI supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (31). The cells were plated at 10^5/well in 12-well dishes (Falcon) and transfected with 1.2 μg of DNA/well using SuperFect according to the manufacturer's protocol (Qiagen). The internal control plasmid pRL-TK (Promega) was co-transfected in all transfection experiments. After 48 h of transfection, the cells were harvested, and luciferase assays were performed using the Dual-Luciferase kit (Promega). The HBV transfection experiment was carried out as described previously (8).

Primer Extension Assay—The primer to map the C gene transcripts was 5′-GGTGAGCAATGCTCAGGAGACTCTAAGG-3′, which corresponds to coordinates 2024–2051 of the HBV genomic sequence. The reaction was performed as previously described (32). Briefly, The HBV DNA pWTD containing the wild type HBV genome (*adw2*) was co-transfected with the nuclear receptors into Huh7 hepatoma cells using the calcium phosphate precipitation method. The pXGH vector served as an internal control for the normalization of transfection efficiency. After 48 h of transfection, the RNA was isolated for the primer extension assay (32).

GST Pull-down Assay—The GST-TR4 fusion protein and GST protein were purified by glutathione-Sepharose beads according to the manufacturer's instructions (Amersham Biosciences). HNF4α was synthesized using the *in vitro* TNT translation system (Promega) in the presence of [^{35}S]methionine, and 5 μl of HNF4α protein was incubated with GST-TR4, TR4-N-DBD, TR4-LBD, or GST beads for the GST pull-down assay as described previously (22).

Co-immunoprecipitation of TR4 and HNF4α Proteins—COS1 cells were cultured in Dulbecco's modified Eagle's medium and co-transfected with 10 μg of pCMX-TR4 and either 10 μg of pCDNAI or pCDNAI-HNF4α. 48 h after the transfection, the cells were lysed in immunoprecipitation buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, and 0.5% Nonidet P-40) containing 1 μg/ml protease inhibitors (Roche Molecular Biochemicals) on ice for 30 min. The cellular lysates were centrifuged at 12,000 × *g* for 10 min. The protein concentration was measured using the Bradford reagent (Bio-Rad), 500 μg of protein was precleared using protein A/G-Sepharose beads (Santa Cruz Biotechnology), and then the mixture was immunoprecipitated with either an anti-TR4 monoclonal antibody or normal mouse IgG (1:100) at 4 °C overnight. Protein A/G beads were added, and the protein lysates were rotated at 4 °C for another hour. The immunocomplexes bound by beads were pelleted and washed with immunoprecipitation buffer three times and then subjected to SDS-PAGE for Western blot analysis using a goat anti-HNF4α polyclonal antibody (Santa Cruz Biotechnology). The results were visualized by enhanced chemiluminescence (ECL). The same blot was stripped in 62.5 mM Tris-HCl (pH 6.8), 2% SDS at 65 °C for 30 min and then blotted with an anti-TR4 monoclonal antibody.

RESULTS

TR4 Protein Binds Specifically to the TR4RE-HBV Consensus Site of the HBV Core Promoter—Previous studies showed that the activity of the HBV core promoter could be differentially regulated by RXRα or PPARα via recognizing consensus binding sites on the HBV core promoter (13, 14). Our previous data also showed that the TR4 has a better affinity than RXRα to recognize the DR1-HRE in the promoter of the cellular retinol-binding protein II gene (21). To examine whether TR4 can bind to the potential DR1-HRE (named TR4RE-HBV, nucleotide coordinates 1751–1778 of the HBV *ayw* DNA sequence) on the HBV core promoter (13), the electrophoretic mobility shift assay (EMSA) was performed with *in vitro* translated TR4 protein, using radiolabeled TR4RE-HBV oligonucleotides as probes. As shown in Fig. 1, no specific supershift complex was seen with an anti-TR4 monoclonal antibody when using mock lysates (Fig. 1, *lanes 1* and *2*). In contrast, the TR4-CpFL supershift complex was observed in the presence of an anti-TR4 antibody using *in vitro* translated TR4 lysates (*lanes 3* and *4*). Moreover, when we replaced wild type TR4RE-HBV with various mutants, we found that TR4 could only bind specifically to the wild type TR4RE-HBV, but not to other mutated TR4RE-HBVs, except for the M3 mutant, which has marginal binding (*lanes 5–10*). The M1 double mutant with nucleotides changed from A to T (1765) and G to A (1767) represents the HBV mutant frequently found in the patients with chronic hepatitis (33). TR4 was not able to bind to the M1 and M2 mutants (*lanes 5* and *6*), indicating that the second mutated nucleotide of the DR1-AGGTCA motif is essential for TR4 binding to the TR4RE-HBV. In addition, a TR4-specific antibody can supershift the TR4·TR4RE-HBV complex (*lane 4*), and this binding can be

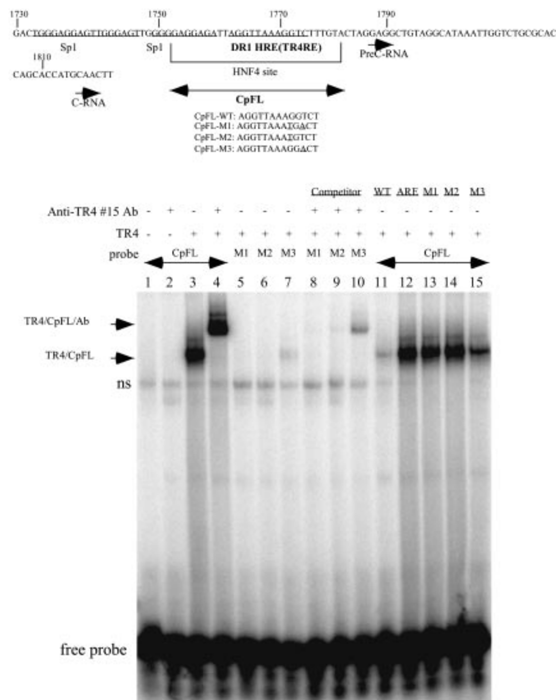

FIG. 1. **TR4 binds to the CpFL element of the HBV core promoter *in vitro*.** TR4 proteins were *in vitro* synthesized in TNT reticulocyte lysates (Promega) and then incubated with CpFL or CpFL mutant probes for 30 min in the presence or absence of an anti-TR4 monoclonal antibody or various 100-fold unlabeled probes as indicated. The reaction mixtures were resolved on a 5% acrylamide native gel, and results were visualized by autoradiography. *ARE* denotes the androgen response element.

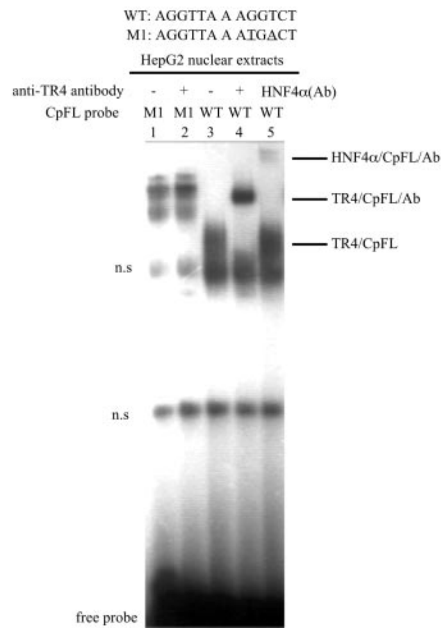

FIG. 2. **The nuclear orphan receptor TR4 is an endogenous DR-1 binding protein in HepG2 cells.** The CpFL probe containing the DR-1 site (nucleotide coordinates 1751–1780) or the M1 mutant probe were incubated with 10 μg of protein of nuclear extracts isolated from HepG2 cells. The EMSA reaction was carried out at room temperature for 30 min in the presence or absence of anti-TR4 or anti-HNF4α antibody. The reaction complexes were analyzed on 5% acrylamide with 0.25× TBE gels, and the results were visualized by autoradiography.

reduced in the presence of 100-fold unlabeled TR4RE-HBV probe but not the probe with an androgen response element probes or TR4RE-HBV mutant probes (*lanes 11–15*), suggesting that the binding between TR4 and TR4RE-HBV is specific.

To further examine whether endogenous TR4 protein can interact with the TR4RE-HBV, nuclear extracts isolated from human HepG2 cells were used in the same EMSA. As shown in Fig. 2, no supershift complex was seen when the nuclear extracts were incubated with the double-mutant M1 probe in the presence of an anti-TR4 antibody (Fig. 2, *lanes 1* and *2*). The wild type TR4RE-HBV probe clearly binds with TR4 as indicated by the TR4·TR4RE-HBV·antibody supershift complex (*lanes 3* and *4*). This result shows that TR4 protein is present in liver cells and interacts with TR4RE-HBV, suggesting that this interaction may play a role in regulating HBV core promoter activity.

TR4 Enhances the Activity of a Luciferase Reporter Linked to TR4RE-HBV in Liver HepG2 and Non-liver H1299 Cells—Our previous data showed that the DR1 consensus sequence (RXRE) from the promoter of cellular retinol-binding protein II could be recognized and suppressed by TR4 (21). We were interested in determining whether the binding of TR4 to the TR4RE-HBV could also allow TR4 to regulate HBV core promoter activity. A reporter construct containing four synthetic copies of the TR4RE-HBV, CpFL(4)Luc, including the HNF4α consensus binding site, was used to determine the role of TR4 (13). CpFL(4) plasmids were co-transfected with either the parental vector or a TR4 expression vector in liver HepG2, HepG2.1, and non-liver H1299 cells. TR4 can transactivate CpFL(4)Luc activity in a dose-dependent fashion in HepG2, HepG2.1, and H1299 cells (Fig. 3, *A*, *B*, and *D*). To confirm that the increase in reporter activity is not due to nonspecific activation by TR4, we also used the TR4 deletion mutants and VDR in this reporter assay. As shown in Fig. 3 (*A–C*) the activity of the reporter was only induced in the presence of full-length functional TR4, but not in VDR or the presence of TR4 mutants lacking the C terminus or N terminus, suggesting that the CpFL (TR4RE-HBV) elements of the core HBV promoter may be sufficient for TR4 to regulate HBV core promoter activity.

TR4 Directly Suppresses HBV Core Promoter Activity through the TR4RE-HBV Site—The EMSA and reporter assay with CpFL(4)Luc suggest that TR4 may have a significant role in the transcriptional regulation of the HBV core promoter. However, the functional role of TR4 in a synthetic promoter context does not necessarily reveal TR4 action in the natural HBV core promoter context. We therefore examined the effects of TR4 on a reporter construct containing the whole context of the HBV genome sequence (CpLuc) (13). Unexpectedly, TR4 suppressed CpLuc reporter activity in HepG2 cells (Fig. 4*A*). In contrast, co-transfection of another nuclear receptor, HNF4α, induced CpLuc reporter activity, consistent with an earlier report (13). To confirm that the suppression was mediated by TR4 through binding to the TR4RE-HBV of the HBV core promoter, we also used CpM2Luc containing the mutated TR4RE-HBV to analyze TR4 suppression (13). As shown in Fig. 4*B*, TR4 suppression of the reporter activity was abolished by mutation of the TR4RE-HBV site. In contrast, HNF4α was able to induce mutant CpM2Luc reporter activity, possibly due to the presence of another functionally intact HNF4α-binding site (*lane 4 versus 6*) (13, 34).

Because the responsiveness of the HBV core promoter to TR4 was abrogated by this mutation, it is possible that the TR4-mediated suppression of the HBV core promoter is mainly dependent upon the binding between TR4 and the TR4RE-HBV. Because earlier reports and our current data show that both TR4 and HNF4α can bind to the TR4RE-HBV site, which is overlapped with the HNF4α consensus binding site (13), we postulated that TR4 may exert its repressive effects on the core promoter through competition with other nuclear receptors such as HNF4α for binding to TR4RE-HBV. We therefore ex-

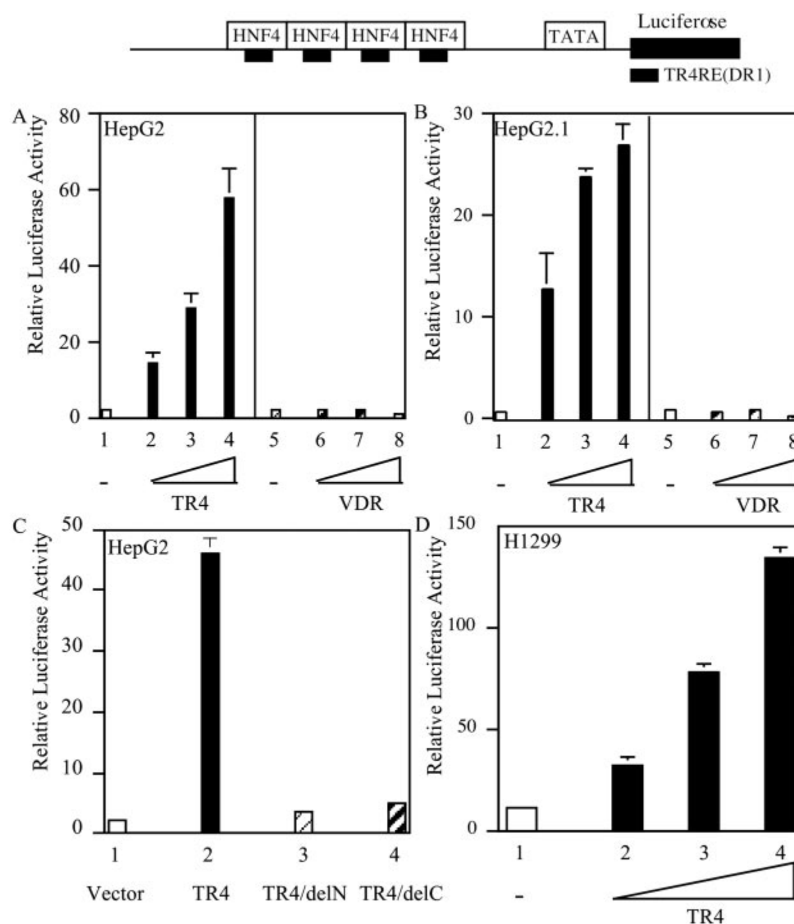

FIG. 3. **Effects of TR4 on a reporter containing the CpFL(DR1) element of the HBV core promoter.** *A*, HepG2 cells were co-transfected with 500 ng of CpFL(4)Luc reporter and increasing amounts of TR4 or VDR (100, 200, and 400 ng). The cells with VDR transfection were treated with 100 nM vitamin D for 24 h, then harvested for the luciferase assay. The luciferase assay was performed as described under "Experimental Procedures." *B*, HepG2.1 cells were transfected with CpFL(4)Luc and TR4 or VDR, treated, and harvested as in *A*. *C* and *D*, HepG2 cells and H1299 cells were co-transfected with CpFL(4)-Luc and the full-length TR4, N/C-terminal deletion of TR4 (TR4/delN and TR4/delC). The reporter assay was carried out as described in *A*. Data represent the means ± S.E. of three independent experiments.

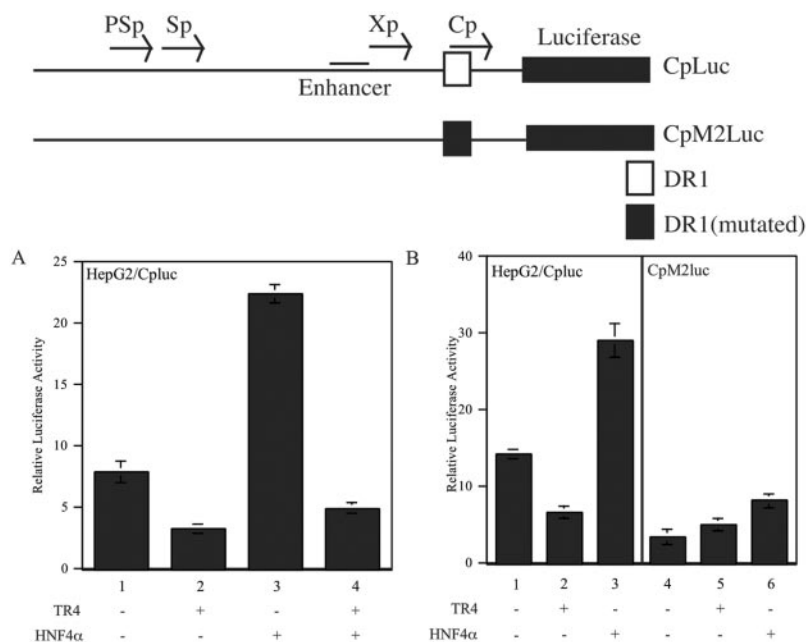

FIG. 4. **TR4 represses HBV core promoter activity.** *A*, HepG2 cells were co-transfected with 750 ng of CpLuc and 250 ng of TR4 or HNF4α vectors or the combination of these two nuclear receptors. The luciferase activity was measured 48 h after the transfection. *B*, the reporters CpLuc (750 ng) or CpM2Luc were co-transfected with TR4 (250 ng) or HNF4α (250 ng) as indicated, then harvested for the luciferase activity 48 h after the transfection. Data represent the mean ± S.E. of three independent experiments. *PSp*, *Sp*, *Xp*, and *Cp* represent the promoters of the genes encoding the HBV surface antigen, X protein, and core/e antigens, respectively.

amined their potential mutual influence on the HBV core promoter. When we co-transfected equal amounts of HNF4α and TR4 with the CpLuc reporter in HepG2 hepatoma cells, TR4 repressed HNF4α-induced HBV core promoter activity (Fig. 4*A*, *column 3 versus 4*), suggesting that HNF4α and TR4 may compete for the same consensus binding site on the HBV core promoter. Similar suppression of HNF4α-mediated transactivation also occurred when we used another reporter containing the HNF4α binding site found in the HBV enhancer I/X promoter overlapped with enhancer I (XpHNF4αLuc, HBV coordinates 1133–1159, data not shown) (13).

Using EMSA with *in vitro* translated TR4 and HNF4α in the presence of [^{32}P]TR4RE-HBV, we also found that the binding between [^{32}P]TR4RE-HBV and TR4 protein was suppressed by the addition of HNF4α (Fig. 5). Fig. 5 demonstrates that TR4 and HNF4α can both bind to the TR4RE-HBV site, and the DNA binding activity of TR4 was markedly reduced in the presence of HNF4α. In contrast, we did not observe any change

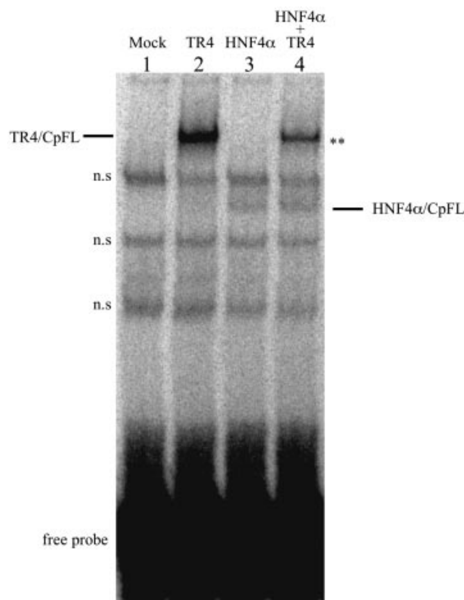

FIG. 5. **HNF4α suppresses TR4 binding to the CpFL element of the HBV core promoter *in vitro*.** TR4 and HNF4α proteins were *in vitro* synthesized in TNT reticulocyte lysates (Promega). 1 μl of TR4 protein was then incubated with CpFL probes for 15 min in EMSA reaction buffer (10 mM Hepes, pH 7.9/6% (v/v) glycerol, 2% (v/v) Ficoll, 100 mM KCl, 0.5 mM EDTA, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol) in the presence or absence of the HNF4α protein. The reaction mixtures were resolved on a 5% acrylamide native gel, and results were visualized by autoradiography. The *double asterisks* indicate that the repression of TR4 DNA by HNF4α.

in HNF4α binding when we added TR4, implying that the simple competition may not fully account for TR4-mediated repression of HNF4α function. This raises the possibility that TR4 may directly block HNF4α transactivation through protein-protein interactions with HNF4α instead of competing for DNA binding sites. Together, Figs. 4 and 5 suggest that TR4 can bind to the same TR4RE-HBV site as well as interact with HNF4α to influence HBV core promoter expression.

Interaction between HNF4α and TR4—The data shown above suggest that TR4 may regulate the transcriptional activity of HNF4α by a mechanism that is independent from the competition for DNA binding. To prove this hypothesis, we constructed a plasmid encoding GAL4-HNF4α fusion protein and examined the effect of TR4 expression on HNF4α-mediated transactivation in the GAL4 one-hybrid system (35). In this assay, the transcriptional activity of GAL4-HNF4α is independent of the binding of HNF4α and TR4 to their cognate binding site, DR1-HRE. If TR4 can directly regulate HNF4α transcriptional activity, we should see the activity of GAL4-HNF4α would be significantly changed in the presence of TR4 expression. HepG2 cells were co-transfected with GAL4-HN4α and the pG4LUC reporter in the presence or absence of the expression of the TR4 N- or C-terminal deletion of TR4 mutants. As the results show in Fig. 6*A*, the reporter activity with GAL-HNF4α alone was increased about 3-fold, which was similarly shown by the previous study (35). Interestingly, the expression of TR4 significantly increased the GAL4-HNF4α-mediated transcriptional activation (Fig. 6*A*), indicating that TR4 can directly regulate the transcriptional activity of HNF4α, which is independent of the event of the DNA binding and possibly mediated by protein-protein interactions. As expected, the PG4LUC reporter activity with the GAL4 and TR4 was not increased at any significant level (Fig. 6*A*, *columns 1 versus 6*), suggesting TR4 influences HNF4α transcriptional activity in a specific manner. Nevertheless, it is not clear how this regulation involving the interaction between TR4 and HNF4α would result in either activation or suppression of HNF4α function. In contrast, the N- or C-terminal deletion of TR4 mutants did not show any effects on the GAL4-HNF4α-mediated transactivation, suggesting that these two regions of TR4 protein may be required for TR4 action in this regulation.

Next, we hypothesized that TR4 may directly regulate HNF4α transcriptional activity through interacting with HNF4α. To test this hypothesis, we applied the GST pull-down assay to determine the potential interaction between HNF4α and TR4. As shown in Fig. 6*B*, HNF4α protein was pulled down and detected by SDS-PAGE from the mixture of ^{35}S-labeled *in vitro* translated HNF4α protein incubated with GST-TR4, GST-TR4-N-DBD, or GST-TR4-LBD fusion protein immobilized on glutathione-linked Sepharose beads. Fig. 6*B* (*lane 3*) clearly demonstrated that HNF4α interacts with TR4 *in vitro*. However, the interaction of the deletion mutants of GST-TR4 fusion protein with HNF4α is much weaker, suggesting that instead of direct competition for binding to the TR4RE-HBV, TR4 may interact with HNF4α, which could possibly result in the repression of HNF4α function. To further confirm this interaction in liver cells, we co-transfected TR4 and HNF4α into COS1 cells and used a co-immunoprecipitation assay to immunoprecipitate the TR4 and HNF4α complex by an anti-TR4 monoclonal antibody. As shown in Fig. 6*C* (*lane 4*), we can clearly observe HNF4α bands in the precipitated complex by Western blotting when co-expressing TR4 and HNF4α proteins in COS1 cells. This result suggests that TR4 can bind to HNF4α.

In summary, the results presented above demonstrate that TR4 is capable of regulating HNF4α transcriptional activity by interacting with HNF4α. Also, the full-length of TR4 is essential for this regulation. We further compared the effects of TR4 on HNF4α-mediated transactivation on the CpLUC reporter with deletion mutants of TR4 in HepG2 cells using transient transfection assay (Fig. 6*D*). As expected and similar to the result shown in Fig. 4*A*, TR4 suppresses HNF4α-mediated transactivation (Fig. 6*D*, *column 3 versus 4*). However, the N- or C-terminal deletion of TR4 did not exhibit similarly suppressive effects as those of the full-length TR4 did (*column 4 versus 7* and *8*), implying that these two domains are essential in the protein-protein interaction of TR4 and HNF4α, and this interaction is required for the repressive action of TR4 on the transcriptional activity of HNF4α.

Suppression of HBV Pre-core and Core mRNA Expression by TR4—To further confirm the suppression of HBV core promoter activity by TR4, we applied a primer extension assay to see if TR4 can also influence mRNA expression of two HBV transcripts from the HBV core promoter. The first transcript is pre-C mRNA, which is the precursor of HBeAg; the other transcript is C mRNA, serving as the template for reverse transcription (6, 36). HBV RNA extracted from Huh-7 after the co-transfection of TR4 and HBV vectors was mapped with radiolabeled HBV primer at position 2024–2051 of the HBV genome (8). As seen in Fig. 7, the expression of both pre-C and C mRNAs was decreased in the presence of TR4. This result therefore confirms the reporter assays showing that TR4 can suppress HBV core promoter transcription via binding to the TR4RE-HBV site on the HBV core promoter.

DISCUSSION

Earlier studies have identified two consensus DR1 binding sites for nuclear receptors in the HBV promoter. One such site (TGAACCTTTACCC, nucleotide coordinates 1138–1150), is located in the core domain of enhancer I and is recognized by HNF4α, COUP-TF1, and RXRα-PPARα (37). The other site (AGGTTAAAGGTCT, nucleotide coordinates 1757–1769) is located in the core promoter that can be recognized by HNF4α

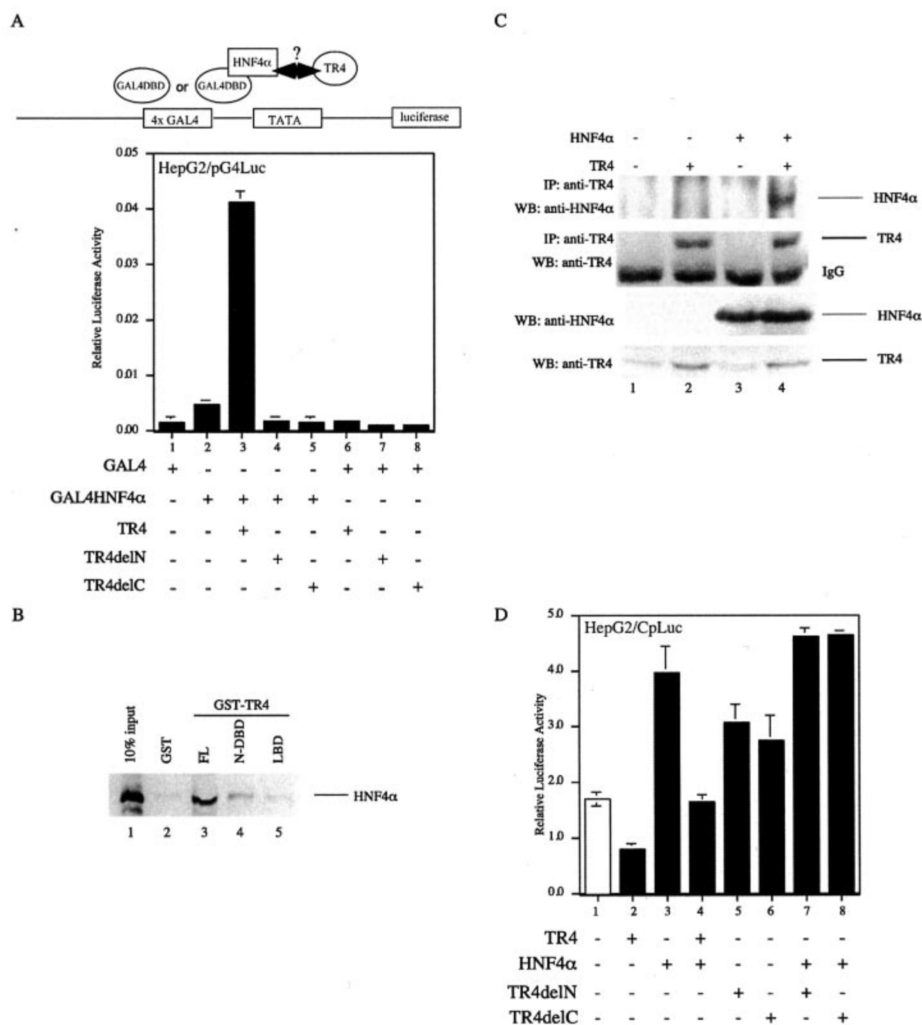

FIG. 6. **Interaction between TR4 and HNF4α.** *A*, diagrammatic representation of the GAL4 hybrid assay. This assay was used to test the effect of TR4 on HNF4α-mediated transcriptional activity. HepG2 cells were co-transfected PG4LUC, which contains four tandem repeats of the GAL4-binding sites, with GAL4-HNF4α, TR4, or TR4 deletion mutants. *B*, *in vitro* synthesized [^{35}S]methionine-labeled HNF4α protein was incubated with GST, GST-TR4, GST-TR4-N-DBD, and GST-TR4-LBD fusion protein bound to glutathione-Sepharose beads. The beads were extensively washed after the reaction and applied to 12% SDS-PAGE followed by autoradiography. *C*, COS1 cells were transfected pCMX-TR4 with either parental vector or pCDNAI-HNF4α. After the transfection, the cells were cultured for 48 h, lysed for the immunoprecipitation assay, and the HNF4α signal was detected by Western blotting with a goat anti-HNF4α antibody. The results were visualized by enhanced chemiluminescence (ECL). Then, the same blot was stripped and blotted with an anti-TR4 monoclonal antibody. *D*, HepG2 cells were transfected CpLUC with HNF4α, TR4, or TR4 deletion mutants as described in Fig. 4*A*.

and TR4 as described in current studies (13). The detailed mechanisms of how these nuclear receptors modulate HBV gene expression via these two DR1 sites remain unclear. Our data strongly suggest that TR4 preferentially binds the DR1 site of the core promoter, because TR4 suppression is abrogated if this site is mutated (Fig. 4*B*). The selective binding of TR4 to the 2nd DR1 site may be a key step for TR4 to prevent other nuclear receptors from binding to this site, thereby repressing transcription. However, we cannot rule out the possibility that TR4 may also regulate HBV gene expression through the DR1 site of enhancer I, because our data indicate that TR4 can recognize this site (data not shown). Moreover, because TR4 binding to the 2nd DR1 site is prevented by two mutations ((1756 (A to T) and 1767 (G to A)), which are frequently found in patients with chronic hepatitis, one may speculate that HBV may utilize these two mutations to escape suppression mediated by TR4 and/or other transcriptional repressors. Alternatively, creation of new mutations at these consensus DR1 sites may be able to increase the opportunity for other modulators to regulate HBV expression via these mutated DR1 sites (32).

TR4 is highly homologous with another orphan receptor, TR2 (15). Earlier studies indicated that TR2 and TR4 could modulate the expression of a set of genes through binding to the same HREs (19, 38). Interestingly, Yu *et al.* (14) reported that TR2 could only repress pre-C RNA, but not C RNA expression. Our current data show that TR4 can repress both pre-C RNA and C RNA expression. Moreover, compared with the marginal suppression of TR2 on CpLuc reporter activity, TR4 has a much stronger suppressive effect on CpLuc reporter activity (data not shown), suggesting that TR4 may play a significant role in this regulation. Also, our data suggest that simple competition for binding to the DR1 site by these two nuclear receptors may not fully account for their distinct regulation of HBV core gene expression, because both nuclear receptors regulate target genes through binding to the DR sites (19, 38). The detailed mechanisms underlying the distinct regulation of HBV gene expression by these two closely related orphan receptors remain unclear. The interaction of TR4 and HNF4α suggests that the TR4 affects HBV gene expression at multiple levels, most importantly, including the DNA binding and protein-protein interactions. Nevertheless, previous reports also suggested that COUP-TF could differentially modulate target gene expression via similar HREs. For example, COUP-TF1 can induce the 7α-hydroxylase promoter activity via the DR4 binding site and repress myosin heavy chain promoter activity via a similar DR4 binding site (39). Similar differential modulation of target gene expression also occurred with TR4, via binding to similar DR1 binding sites (21, 23).

The contrasting effects of TR4 on the synthetic TR4RE-HBV *versus* the entire HBV promoter (Fig. 3 *versus* 4) suggest that promoter context, especially sequences other than the TR4RE-HBV, may also play important roles for TR4 to modulate HBV core promoter activity. It is possible that sequences flanking the TR4RE-HBV may recruit one or more repressors that can cooperate with TR4 to form a protein complex to repress HBV core promoter. In addition, we have examined the effects of TR4 on HBV core promoter activity in non-liver cells and found that TR4 was able to induce the luciferase activity of the Cpluc

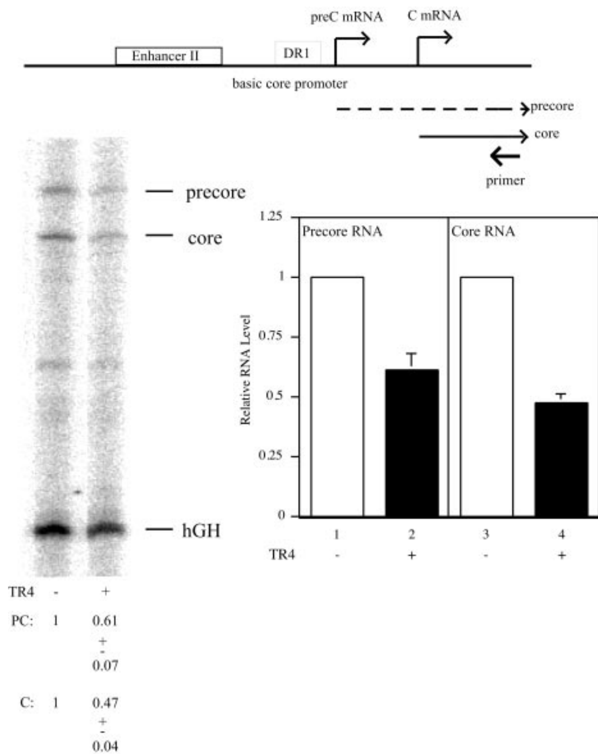

FIG. 7. **Primer extension analysis of the effect of TR4 on HBV pre-core and core RNA expression.** HBV RNA isolated from Huh-7 cells transfected with HBV DNA and TR4 or the parental vector of TR4 as indicated was analyzed by primer extension assay as described under "Experimental Procedures." Briefly, the products of the primer extension were subjected to electrophoresis on an 8% sequencing gel. The locations of the core, pre-core, and human growth hormone (*hGH*) RNA are indicated. The hGH served as an internal control. The results were visualized by autoradiography and quantified by Sigma scan software. The value of the quantification represents three independent experiments.

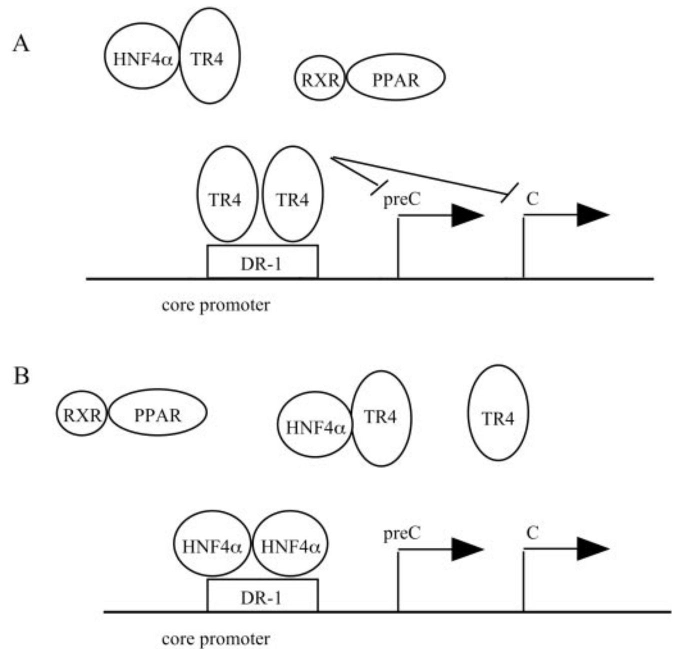

FIG. 8. **Schematic diagram of the regulation of the HBV core promoter by TR4 through the DR1 (TR4RE-HBV) site.** This figure depicts a simple model of the HBV core promoter and the regulation of this promoter via TR4RE-HBV by TR4. *A*, The binding of TR4 to the TR4RE may restrict the accessibility of this site to other essential nuclear receptors for transcriptional activation. Also, the interaction of HNF4α and TR4 inhibits HNF4α transcriptional activity and results in the suppression of the HBV core promoter activity. *B*, during natural infection, by an unknown mechanism, the interaction of HNF4α and 2R4 may prevent TR4 from binding to the DR1 site (Fig. 5). Therefore, this regulation can relieve TR4-mediated repression and allow HNF4α or other nuclear receptors to proceed to the transcriptional activation.

reporter in H1299 cells (data not shown). This interesting finding implies that some cellular factors in liver cells may modulate the transcriptional activity of TR4 in the regulation of the HBV core promoter. However, the detailed mechanisms of this distinct regulation by TR4 remain unclear.

TR4 may modulate its target gene expression via two distinct mechanisms. First, TR4 can suppress target gene expression via competition with other nuclear receptors, such as RXRα (DR1), VDR (DR3), and retinoic acid receptor α (DR5), for the same consensus HRE sites (21, 22). Previous studies showed that ligand-activated RXRα/PPARα or PPARγ can increase HBV core promoter activity through the TR4RE-HBV site (DR1) (13, 14). Therefore, it appears that TR4 may compete for the same binding site with these nuclear receptors resulting in repression of HBV core promoter activity, which this regulation does not involve in any interaction between TR4 and other nuclear receptors (21, 22). Second, TR4 can also modulate gene expression via protein-protein interactions with other nuclear receptors. For example, TR4 can suppress androgen target genes through interactions with the androgen receptor (40). In the current study, we found TR4 cannot only bind to the same DR1 site on HBV core promoter as HNF4α (Fig. 5), but also interact with HNF4α (Fig. 6, *B* and *C*). The TR4-mediated repression resulted from this interaction was confirmed by using TR4 deletion mutants in several parallel experiments and may further support that there might be multiple mechanisms by which TR4 proceeds in this repressive regulation. Previous studies showed that TR2, a close member of TR4 family, and RIP140 (receptor-interacting protein 140), one of TR4-interacting proteins, can interact and recruit histone deacetylases for gene silencing (22, 41, 42). It is possible that TR4 may carry out this repression through similar mechanisms, which would be an interesting direction for our future study to test the involvement of histone deacetylases in this regulation.

On the other hand, in HBV-infected liver cells, the repressive function of TR4 may be unfavorable to the HBV replication because the core promoter controls the transcription of the template C RNA for viral DNA synthesis. We speculate that the functional association of TR4 and HNF4α could be a unique way to relieve TR4-mediated repression, because HNFα can reduce TR4 binding to the DR1 site through either competition for DNA binding or protein-protein interactions (Fig. 5). The underlying mechanisms of how these nuclear receptors differentially influence the transcription activation of HBV genes during infection will require more extensive study.

As summarized in Fig. 8, TR4 can directly bind to the TR4RE-HBV and therefore prevent other nuclear receptors from activating the HBV core promoter. Alternatively, TR4 can interact with nuclear receptors, such as HNF4α, to form an inactive protein complex. Either of these two activities may result in the suppression of HBV core promoter.

In conclusion, this study provides evidence that TR4 is a transcriptional repressor of the HBV core promoter. The interaction of TR4 and HNF4α further extends the scope of nuclear receptor regulation of the HBV core promoter. The transcriptional activation of the core promoter is a key step to initiate the life cycle of HBV during the infection (34). By identifying of physiological ligand(s) for TR4, we may be able to control agonist or antagonist binding to TR4 to modulate HBV gene expression, which may provide a novel strategy to develop anti-HBV drugs.

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