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Transcriptional Regulation of Human Eosinophil RNase2 by the Liver-Enriched Hepatocyte Nuclear Factor 4

Hsiu-Yu Wang, Po-Chun Ho, Chung-Yu Lan, and Margaret Dah-Tsyr Chang*

Institute of Molecular and Cellular Biology & Department of Life Science, National Tsing Hua University, No. 101, Sec. 2, Kuang-Fu Rd., Hsinchu, Taiwan 30013, Republic of China

ABSTRACT

Human eosinophil-derived neurotoxin (EDN, RNase2) and eosinophil cationic protein (ECP, RNase3) sequences possess as high as 92% identity in their promoter regions. The major difference within this region is a 34-nucleotide (34-nt) segment appeared only in the *edn* promoter. In addition, six discrete segments existed in the regulatory regions of both *edn* and *ecp*. Our previous study indicated that the 34-nt segment is responsive for higher transcription activity of *edn* in comparison with *ecp*, via binding to transcription activator Sp1. In this study, the roles of the six discrete segments in transcription regulation were investigated and the −350/−329 region (*edn*R2) was shown to be involved in the regulation of *edn* expression. When the *edn*R2 segment of *edn* was replaced with that of *ecp*, a significant decrease in *edn* promoter activity was detected. Supershift, chromatin immunoprecipitation, and DNA affinity precipitation assays further showed that a transcription factor HNF4 bound to the *edn*R2 region of *edn* promoter in vitro. Interestingly, HNF4 overexpression resulted in the reduction of *edn* promoter activity in HepG2 cells, due to involvement of both *edn*R2 and the 34-nt regions, and direct interaction between HNF4 and Sp1, which abolishes Sp1 binging to the 34-nt segment. Moreover, when the Sp1 was depleted in the cell, overexpressed HNF4 enhanced *edn* promoter activity. Our results provide novel mechanisms for HNF4 function as an activator to regulate *edn* promoter activity, which account for differential transcription regulation of human eosinophil RNases. J. Cell. Biochem. 106: 317–326, 2009. © 2008 Wiley-Liss, Inc.

KEY WORDS: EOSINOPHIL CATIONIC PROTEIN; EOSINOPHIL-DERIVED NEUROTOXIN; HEPATOCYTE NUCLEAR FACTOR

Human ribonuclease A (RNase A) superfamily is consisted of eight members including the pancreatic ribonuclease (RNase1), the eosinophil-associated RNases (EARs) containing eosinophil-derived neurotoxin (EDN or RNase2) and eosinophil cationic protein (ECP or RNase3), RNase4, the angiogenins (ANGs or RNase5), RNase6 (k6), RNase7, and RNase8 [Sorrentino and Libonati, 1997; Zhang et al., 2003; Yang et al., 2004; Cho et al., 2005; Nitto et al., 2005]. Interestingly, the genes encoding these human RNases are all localized to the q24–q31 region of chromosome 14 [Cho et al., 2005] due to a series of gene duplication and evolution events.

Eosinophils participate in the pathogenesis of parasitic disease, but their role in promoting protection against infection or host defense is controversial [Klion and Nutman, 2004; Dyer and Rosenberg, 2006]. The concentration of serum ECP and EDN, and urinary EDN were significantly higher in asthmatic children as compared with the controls [Zimmerman et al., 1993; Oosterhoff et al., 1995; Kim et al., 2007]. The cDNA sequence of *ecp* is 84.2% identical to that of *edn*, and the identity of amino acid sequences between ECP and EDN is as high as 69.6%. Both ECP and EDN are expressed in activated eosinophils [Gleich et al., 1986; Rosenberg et al., 1989], but EDN is also reported to be expressed in other tissues such as liver [Sorrentino et al., 1988], spleen [Yasuda et al., 1990], and kidney [Mizuta et al., 1990].

Sequence identity between the coding regions of human *edn* and *ecp* is shown to be as high as 85%, while the sequence identity of the 1 kb-regulatory regions upstream of human *edn* and *ecp* genes is

Abbreviations used: ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; HNF4, hepatocyte nuclear factor 4; Sp1, SV-40 protein 1; MAZ, Myc-associated zinc finger protein; ChIP, chromatin immunoprecipitation; EMSA, electromobility shift assay; DAPA, DNA affinity precipitation assay; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; LEF-1, lymphoid enhancer factor-1.

Hsiu-Yu Wang and Po-Chun Ho contributed equally to this work.

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*Correspondence to: Dr. Margaret Dah-Tsyr Chang, Institute of Molecular and Cellular Biology & Department of Life Science, National Tsing Hua University, Taiwan 30013, Republic of China. E-mail: lscmdt@lige.nthu.edu.tw

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even higher up to 92%. Two binding sites for the transcriptional factors C/EBP and PU.1 have been revealed in the *edn* promoter regions [van Dijk et al., 1998; Baltus et al., 1999]. A consensus binding sequence for NFAT-1 was also found in the intron region of *edn*, acting as an enhancer for its promoter activity [Handen and Rosenberg, 1997]. In our previous study, the presence of a 34-nt segment enhanced the promoter activity of both *edn* and *ecp* was further identified in HepG2 cells, however, *edn* promoter activity was significantly higher than that of *ecp* under all conditions tested [Wang et al., 2007]. Two transcription factors Sp1 and MAZ can bind to the 34-nt segment and regulate *edn* promoter activity through a competitive manner [Wang et al., 2007]. In addition to the 34-nt segment, some minor differences between the regulatory regions of *edn* and *ecp* promoters were also revealed; these segments in both of these promoter regions were selected for further investigation. In this study, mutagenesis, luciferase reporter assay, EMSA and chromatin immunoprecipitation (ChIP) were used to identify key regulatory motifs in governing the promoter activity, and to investigate whether any novel transcription factor(s) can recognize and bind to these regulatory motifs.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

HepG2 cells (human hepatocellular carcinoma cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS, Biological Industries). All cells were cultured in the foregoing medium with 100 unit/ml penicillin G and 10 μl/ml streptomycin at 37°C in an atmosphere containing 5% CO_2. For transient transfection, the HepG2 cell lines were treated with Transfast (Promega) and applied with electroporation, respectively, according to the manufacturer's instructions. The pcDNA3/HNF4 and pcDNA3/DN-HNF4 expression plasmids were kindly provided by Dr. Todd Leff (Department of Pathology, Wayne State University School of Medicine, Detroit, MI).

RNA interference (RNAi) duplexes directed against Sp1 were purchased from Invitrogen Life Technologies. The targeted sequences used to silence Sp1 was 5′-GCAGACACAGCAGCAA-CAAAUUCUU-3′. Forty nanomolar RNAi was transfected into HepG2 cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen Life Technologies).

PROMOTER ACTIVITY ASSAY

After 48 h transfection with individual reporter plasmids, the cells were washed twice with PBS and lysed by Passive Lysis Buffer (Promega). The cell lysates were centrifuged at 16,500*g* for 1 min at 4°C, and the supernatant was collected. The firefly and *Renilla* luciferase activities were measured by TD-20/20 luminometer (Victor), and the relative activity was calculated by simply dividing luminescence intensity obtained from the assay for firefly luciferase by that of *Renilla* luciferase [Wang et al., 2007].

PREPARATION OF NUCLEAR EXTRACT

Nuclear extracts were prepared by NE-PER nuclear extraction kit (Pierce). Briefly, approximately 5×10^7 of the HepG2 cells were trypsinized, collected, and suspended in 200 μl CER I buffer. After incubation for 10 min on ice, 11 μl CER II buffer was added and incubated for another 1 min on ice, the cytoplasmic and nuclear extracts were separated by centrifugation at 16,500*g* for 5 min at 4°C. Cytoplasmic extracts in the supernatant fraction were transferred into a clean microcentrifuge tube, whereas the pellets were resuspended by adding 100 μl ice-cold NER and the mixture was incubated for 40 min on ice. The mixture was finally centrifuged at 16,500*g* for 10 min at 4°C. Nuclear extracts in the supernatant fraction were transferred into a clean microcentrifuge tube and stored at −80°C until use. Concentrations of proteins in the lysates were determined with BCA kit (Pierce) with bovine serum albumin (fraction V) as the standard.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

Probe annealing was performed by heating 10 μM of each complementary strand of the oligonucleotide to 95°C for 10 min, and then cooling gradually to 25°C over a period of 1 h. The probe was synthesized to span the region 1–6 of the human *edn* or *ecp* promoters. The probes used for EMSA (Table I) were prepared by end-labeling with T4 polynucleotide kinase and [gamma-^{32}P]dATP. The labeled probes were then purified by passing through a Sephadex G-25 spin column (Amersham Pharmacia Biotech). Binding reactions were conducted with [gamma-^{32}P]dATP-labeled probe, 1 μg of poly (dI-dC), 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM EDTA, and 10% glycerol, in a total volume of 20 μl. The reaction mixture was carried out at 30°C for 30 min. For competition experiments, nuclear extracts were incubated with a 100–200-fold excess of double-strand competitor oligonucleotides at 30°C for 30 min before

TABLE I. Oligonucleotides Used for EMSA

Oligo ID↓	Sequence (5′–3′)
*ecp*R2	GCCTTCGTGTCATTTAGTCATT
*edn*R2	GCCTTCATGTACTTTGGTCATT
NF-1	TCTTTTGGAATTTATCCAAATCT
C/EBP	TGCAGATTGCGCAATCTGCA
AP-1	CGCTTGATGACTCAGCCGGAA
GR	AGAGGATCTGTACAGGATGTTCTAGAT
GR mut	AGAGGATCTCAACAGGATCATCTAGAT
ER	GGATCTAGGTCACTGTGACCCCGGATC
LEF-1	CTGCCGGGCTTTGATCTTTGCTTAACAA
LEF-1 mut	CTGCCGGGCTTTGGCATTTGCTTAACAA
XBP-1	TAGGATGACGTGTACAATAA
AML-1	CGAGTATTGTGGTTAATACG
SRY	GTTAACGTAACAATGAATCTGGTAGA
COUP-TF	AGCTTCAGGTCAGAGGTCAGAGAGCT
COUP-TF mut	AGCTTCATATCAGATATCAGAGAGCT
TCF	GGGAAGATCAAAGGGGGGG
AP-2 mut	GATCGAACTGACCGCTTGCGGCCCGT
HNF-4	CTCAGCTTGTACTTTGGTACAACTA
HNF-4 mut	CTCAGCTTCTACTTAGGTACAACTA
M 1-2	AACTTCATGTACTTTGGTCATT
M 3-4	GCAGTCATGTACTTTGGTCATT
M 5-6	GCCTGAATGTACTTTGGTCATT
M 7-8	GCCTTCGCGTACTTTGGTCATT
M 9-10	GCCTTCATACACTTTGGTCATT
M 11-12	GCCTTCATGTGATTTGGTCATT
M 13-14	GCCTTCATGTACGGTGGTCATT
M 15-16	GCCTTCATGTACTTCAGTCATT
M 17-18	GCCTTCATGTACTTTGAGCATT
M 19-20	GCCTTCATGTACTTTGGTAGTT
M 21-22	GCCTTCATGTACTTTGGTCAGG

addition of radiolabeled probes. To identify the transcription factors constituting in the protein–DNA binding complexes, anti-HNF4 (sc-8987, Santa Cruz Biotechnology) was included in the binding reactions. The protein–DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel electrophoresis using 45 mM Tris, 45 mM boric acid, and 1 mM EDTA (pH 8.3). The gel was dried and exposed to X-ray film at −70°C using an intensifying screen.

EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEIN

The inserts of pcDNA3/HNF4 and pcDNA3/dHNF4 were subcloned into pET23a to generate pET23a/HNF4 or pET23a/dHNF4. *E. coli* BL21-CodonPlus® (DE3) was used as the bacterial host to produce fusion proteins. pET23a/HNF4 or pET23a/dHNF4 was individually transformed into *E. coli* BL21-CodonPlus® (DE3) competent cells. Cell extracts containing the recombinant proteins were adjusted to pH 8, passed through a 22 μm filter and applied to an Ni^{2+}-saturated chelating Sepharose column (Amersham Biosciences, Freiburg, Germany) equilibrated with binding buffer (pH 8). Specifically bound proteins were eluted with 250 mM imidazole in binding buffer (pH 8). Protein fractions containing HNF4 or dHNF4 were separated by SDS/PAGE and identified by immunoblotting with the HNF4 specific antibody (Santa Cruz Biotechnology, Heidelberg, Germany), pooled, dialyzed against PBS (pH 7.4) and stored at −80°C until use.

The pBAC-Sp1 expression plasmids were kindly provided by Dr. Ariella Oppenheim (Department of Hematology, The Hebrew University-Hadassah Medical School and Hadassah University Hospital, Jerusalem, Israel). His-Sp1 was produced and purified according to previous report [Gordon-Shaag et al., 2002].

IMMUNOPRECIPITATION

For immunoprecipitation experiments, nuclear extract protein of HepG2 cells was pre-cleared by incubation with 30 μl of protein G PLUS-agarose (Santa-Cruz Biotechnology) on a rotating platform for 3 h at 4°C, followed by 30 s of spin (quickspin) and collection of the supernatant. The supernatants were incubated with 5 μl polyclonal anti-Sp1 or anti-HNF4 antibody (Santa-Cruz Biotechnology) on a rotating platform overnight at 4°C, followed by incubation with 30 μl of protein G PLUS agarose beads for 3 h. The beads were washed three times with PBS containing 0.02% Tween-20, pelleted by 30 s of spin at 4°C, followed Western blot analysis.

WESTERN BLOTTING

After electrophoresis, the proteins were transferred onto a PVDF membrane (GE Healthcare). The membrane was incubated in 3% BSA at 25°C for 1 h prior to incubation overnight with specific primary antibody, followed by secondary antibody HRP-conjugated IgG (1:5,000, Jackson ImmunoResearch) for 2 h. The target proteins were visualized by the ECL system (Pierce).

DNA AFFINITY PRECIPITATION ASSAY (DAPA)

The oligonucleotides representing the region 2 segment and its mutant within the *edn* promoter were biotinylated at the 5′-termini, and then annealed with their complementary strands, respectively. The DAPA was performed by incubating 400 μg of nuclear extract with 20 μg poly (dI-dC) in binding buffer (20 mM HEPES pH 7.6, 10 mM $(NH_4)_2SO_4$, 1 mM EDTA, 1 mM dithiothreitol, 1% Tween-20, 100 mM KCl) that was pre-cleaned with 50 μl streptavidin-agarose beads (Amersham) (17-5113-01) for 1 h at 4°C with gentle rocking, and then the supernatant incubated with 2 μg of biotinylated DNA probe for 4 h or overnight at 4°C with gentle rocking. The protein–DNA complexes incubated with 20 μl streptavidin-agarose beads for 1 h at 4°C with gentle rocking. Beads were collected and washed with binding buffer containing 0.5% Nonidet P-40 for five times. Sample buffer (2×) was added to the streptavidin-precipitated DNA–protein complex, which was then boiled for 10 min to dissociate the complexes. The proteins were resolved by 10% SDS–polyacrylamide gel electrophoresis, followed by Western blot detection with specific antibodies.

CHROMATIN IMMUNOPRECIPITATION (CHIP)

The ChIP assay kit and anti-HNF4 antibody (Santa Cruz, sc-8987) were used according to the manufacturer's instructions. Immunoprecipitations were performed at 4°C overnight with 5 μg primary antibody. Immune complexes were harvested with protein A-Sepharose beads (60 μl/precipitation) as described. Following immunoprecipitation, washing, and purification of DNA, the samples were dissolved in water and used as templates for PCR amplification using two primers: 5′-GCCCTCAGTGGCTCTATTTGTT-3′ and 5′-TGCTGCTCTTTCTGCTATAA-3′. The PCR products were separated on 2% agarose gel and stained by EtBr, and then DNA fragments were observed under UV transilluminator system.

STATISTICAL ANALYSIS

All experiments were performed at least three times, each in duplicate. The data are expressed as the means ± SE.

RESULTS

HUMAN *EDN* AND *ECP* PROMOTERS CONTAIN DISCRETE REGIONS WITH VARIATIONS

The upstream 1-kb sequences between human *edn* and *ecp* share 92% pair-wise identity as determined by ClustalW [Larkin et al., 2007]. In addition to the presence of an evident 34-nt segment located at the −81/−48 region in the *edn* promoter, six separate segments containing at least three nucleotide variations in eight consecutive bases between *edn* and *ecp* sequences from the 1-kb promoter to the intron-1 regions were found [Wang et al., 2007]. From upstream to downstream of the coding region, these segments located at regions correspondent to −560/−541, −350/−329, −262/−238, +44/+64, +101/+116, and +151/+178 in both *edn* and *ecp* were respectively named as R1–R6. Whether any of these 12 short segments, *edn*R1 to *edn*R6 and *ecp*R1 to *ecp*R6, contained regulatory elements for transcription was further investigated.

REPLACEMENT OF *EDN*R2 WITH *ECP*R2 DECREASES TRANSCRIPTION ACTIVITY OF *EDN* PROMOTER

Previous study demonstrated that transcription factors, MAZ and Sp1, competitively bound to the 34-nt segment enhanced the promoter activity of *edn* in HepG2 cells [Wang et al., 2007]. Insertion of the 34-nt motif could increase the promoter activity of *ecp* up to

approximately threefold (Fig. 1A). Alternatively, deletion of the 34-nt segment from *edn* promoter resulted in approximately 70% decrease of *edn* promoter activity (Fig. 1A). These results suggested that in addition to the crucial 34-nt segment, some other regulatory motifs, possibly located within the R1–R6 segments of *edn* and *ecp* might also play important roles. Therefore, each of the *edn*R1 to *edn*R6 segment in *edn* promoter constructed in pGL3-basic plasmid was respectively replaced with the corresponding *ecp*R1 to *ecp*R6 segments to generate *edn*-mutR1, *edn*-mutR2, *edn*-mutR3, *edn*-mutR4, *edn*-mutR5, and *edn*-mutR6 employing site-directed mutagenesis. Two days after transfection in HepG2 cells, luciferase reporter assay was carried out to measure the promoter activity (Fig. 1B). As compared with the control containing wild-type *edn* promoter, the promoter activity of *edn*-mutR1 and *edn*-mutR6 increased approximately 20%, whereas that of *edn*-mutR2 decreased to about 60%. As for the others, *edn*-mutR3 maintained the same promoter activity as the wild-type *edn*, and the promoter activity of *edn*-mutR4 and *edn*-mutR5 remained about 90%. It appeared that *edn*-mutR2 with swapped *ecp*R2 segment showed a significantly lower promoter activity than *edn*, suggesting that this *edn*R2 region might contain putative regulatory motif(s). As *edn*R2 of *edn* Δ (−81/−48) was replaced with the correspondent *ecp*R2 to generate *edn* Δ (−81/−48)-mutR2, the promoter activity dramatically decreased, strongly suggesting that *edn*R2 contained a regulatory transcription element. Interestingly, the promoter activity of *edn* Δ (−81/−48)-mutR2 was close to that of *ecp* (Fig. 1C), indicating that sequence variation in −81/−48 and *edn*R2 regions together also contributed to differential promoter activity between *edn* and *ecp*.

TRANSCRIPTION FACTORS ASSOCIATE WITH THE EDNR2 AND ECPR2 SEGMENTS

To elucidate the role of *edn*R2 in *edn* expression by serving as a recognition site for transcription factors, electrophoretic mobility shift assay (EMSA) was carried out employing oligonucleotide probes containing wild-type sequence of *edn*R2 "GCCTTCATGTACTTTGGTCATT" and that of *ecp*R2 "GCCTTCGTGTCATTTAGTCATT". Each probe used for EMSA was end-labeled with T4 polynucleotide kinase and [gamma-^{32}P]ATP. After incubation with the HepG2 nuclear extracts followed by native-PAGE, one evident major DNA–protein complex (Fig. 2, lane 2) and one minor complex (Fig. 2, lane 6) were observed with the *edn*R2 and *ecp*R2 probe, respectively. Interestingly, each of the complex signals was specifically competed off by 200-fold molar excess unlabeled DNA containing of the same R2 sequence, but unlabeled *edn*R2 and *ecp*R2 probes could not compete off the complex signals with each other. It indicated that the putative proteins binding to *edn*R2 and *ecp*R2 should be different.

POTENTIAL TRANSCRIPTION FACTOR(S) BOUND TO *EDN*R2 ARE IDENTIFIED BY EMSA

The combination of PROMO and Transcription Element Search System (TESS) analyses suggested several putative transcription factor binding sites, including COUP-TF, AML-1, HNF-4, C/EBP, LEF-1, c-Jun (AP-1), NF-1, GR, XBP-1, ER, SRY, TCF and AP-2, in *edn*R2. Among these 13 putative transcription factors, the ones possibly bound to *edn*R2 rather than *ecp*R2 were screened by EMSA. Competition experiments were carried out using 13 individual oligonucleotides each specifically representing the core-binding motif for each transcription factors. Interestingly, the specific *edn*R2–protein complex signal was competed off by adding 200-fold molar excess of the non-radioactive labeled probes containing *edn*R2, and the DNA-binding sequences of COUP-TF, HNF-4, and LEF-1, respectively (Fig. 3A, lanes 2, 5, 6, and 8). The *edn2*-protein complex was not affected by the presence of 200-fold molar excess of each oligonucleotide containing DNA-binding sequence for the

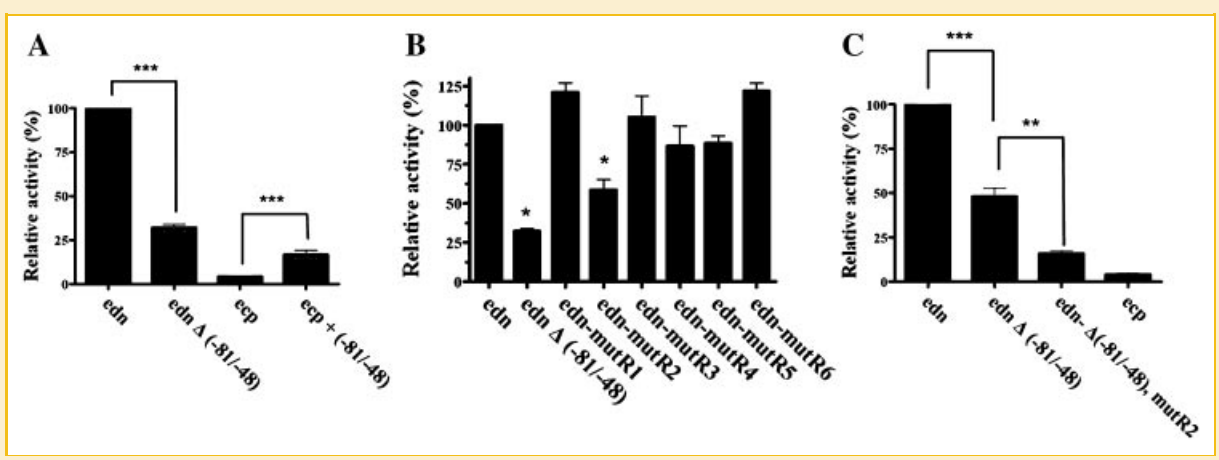

Fig. 1. The role of *edn*R2 in transcriptional activity in HepG2 cells. A: HepG2 cells were transfected with the luciferase reporter plasmid pGL3-*edn* or pGL3-*edn*Δ (−81/−48), pGL3-*ecp*, pGL3-*ecp* + (−81/−48), respectively. B: The HepG2 cells were transfected with luciferase reporter vector containing promoter region deriver form *edn*, *edn* with deletion of the 34-nt segment (*edn* Δ −84/−48), *edn* with mutant −560/−541 region (*edn*-mutR1), *edn* with mutant R2 region (*edn*-mutR2), *edn* with mutant −262/−238 region (*edn*-mutR3), *edn* with mutant +44/+64 region (*edn*-mutR4), *edn* with mutant +101/+116 region (*edn*-mutR5), and *edn* with mutant +151/+178 region (*edn*-mutR6), respectively. C: The HepG2 cells were transfected with luciferase reporter vector containing promoter region deriver form *edn*, *edn* with deletion of the 34-nt segment (*edn* Δ −84/−48), *edn* with mutant R2 region, *edn* with mutant R2 region and deletion of 34-nt segment (*edn*-mutR2, Δ −84/−48), respectively. Luciferase activities were measured using the luciferase assay system. The average values of promoter activities were calculated as described in Materials and Methods Section and obtained from three independent experiments (n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ ANOVA, *t*-test).

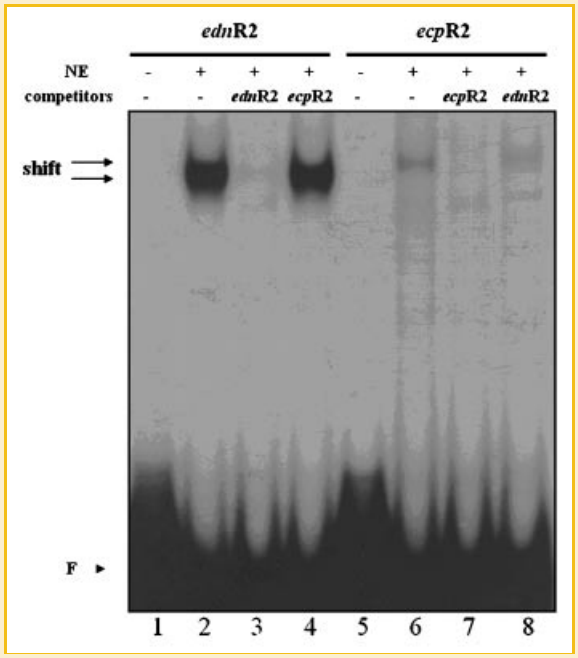

Fig. 2. Transcription factors binding with ednR2 and ecpR2. The labeled probes were incubated with HepG2 nuclear extract, and the DNA–protein complexes were separated by 6% non-denaturing acrylamide gel electrophoresis. Lanes 1–4 contained ^{32}P-labeled *edn*R2 probe. Lanes 5–8 contained ^{32}P-labeled *ecp*R2 probe. Lanes 1 and 5, no nuclear extract, and no competitor oligonucleotide. Lanes 2 and 6, no competitor oligonucleotide. Lanes 3 and 7, a 200-fold of unlabeled *edn*R2 and *ecp*R2 probe as the competitor. Lanes 4 and 8, a 200-fold of unlabeled *ecp*R2 and *edn*R2 probe as the competitor. The specific complex was indicated by the arrow on the left. EMSA was obtained from at least three independent experiments.

other ten transcription factor, nor a negative control of an irrelevant probe (Fig. 3A, lanes 3, 4, 7, and 9–15). To further examine the binding specificity between *edn*R2 with these three putative transcription factors, each probe containing mutations in the core DNA-binding sites of COUP-TF, HNF4, or LEF-1 was used as the competitors in EMSA. The results indicated that *edn*R2–protein complex formation could be specifically rescued in the presence of COUP-TF-m HNF4-m and LEF-1-m (Fig. 3B, lanes 4, 6 and 8). It should be noted that the HNF4 probe utterly competed the complex signal, whereas COUP-TF and LEF-1 probes could only partially compete the signal.

HNF4 RECOGNIZES THE *EDN*R2 SEGMENT

To investigate the involvement of HNF4 in regulation of the *edn* gene, EMSA was performed along with the detection using a polyclonal anti-HNF4 antibody. The presence of anti-HNF4 but not irrelevant antibody could supershift the complex signal, strongly indicating the involvement of HNF4 in the *edn*R2 recognition and the formation of an antibody-HNF4-DNA complex (Fig. 4A). As compared to the data shown in Fig. 3B, the probe with consensus DNA-binding sequence of COUP-TF and LEF-1 also competed off the specific shift with weak signal reduction. However, COUP-TF was not involved in the DNA–protein complex employing DNA affinity precipitation assay (DAPA) technique (data not shown). To map the exact HNF4 binding sequence on *edn*R2, a series of mutagenic EMSA probes containing sequential 2-base pair mutations (M1-2 to M21-22) in the *edn*R2 were used as competitors. As shown in Figure 4B, 200-fold molar excess of the unlabeled probes M9-10, M11-12, M13-14, and M15-16 could not compete with the protein–DNA complex (lanes 7–10), indicating that the mutated DNA bases in these probes were crucial for HNF4 binding to *edn*R2. Therefore,

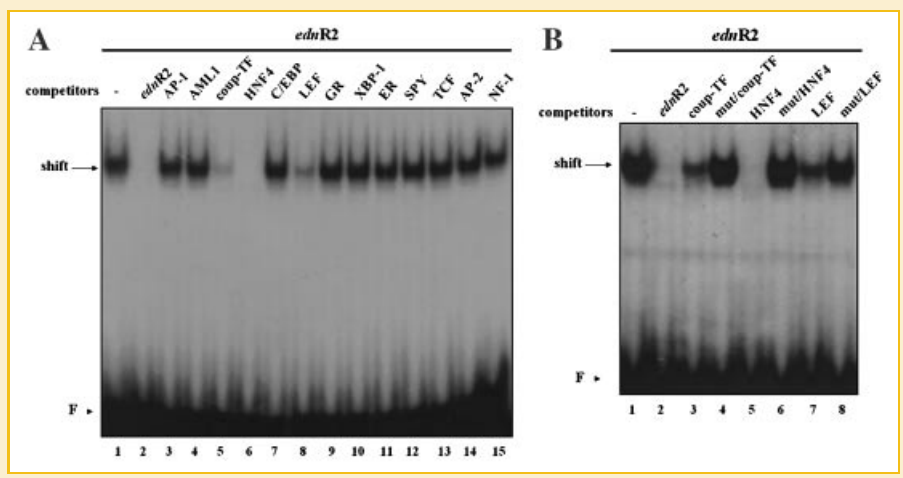

Fig. 3. A HNF4-binding motif present in the ednR2 region. A: The labeled probes were incubated with HepG2 nuclear extract, and the DNA–protein complexes were separated by 6% non-denaturing acrylamide gel electrophoresis. Lane 1 contained no competitor oligonucleotide. Lanes 2–15 contained a 200-fold molar excess of unlabeled oligonucleotide representing *edn*R2, AP-1 binding site (AP-1), AML-1 binding site (AML-1), COUP-TF binding site (COUP-TF), HNF-4 binding site (HNF-4), C/EBP binding site (C/EBP), LEF-1 binding site (LEF-1), GR binding site (GR), XBP-1 binding site (XBP-1), ER binding site (ER), SRY binding site (SRY), TCF binding site (TCF), AP-2 binding site (AP-2), NF-1 binding site (NF-1) probe, respectively. The specific complex was indicated by the arrow on the left. B: The end-labeled *edn*R2 probe was incubated with 20 μg cell nuclear extract proteins from HepG2 cells in the absence of competitor (lane1). Lanes 2–8 contained a 200-fold molar excess of unlabeled oligonucleotide representing *edn*R2, COUP-TF binding site (COUP-TF), mutated COUP-TF binding site (COUP-TF-m), HNF-4 binding site (HNF-4), mutated HNF-4 binding site (HNF4-m), LEF-1 binding site (LEF-1) and mutated LEF-1 binding site (LEF1-m) probe, respectively. The specific complex was indicated by the arrow on the left.

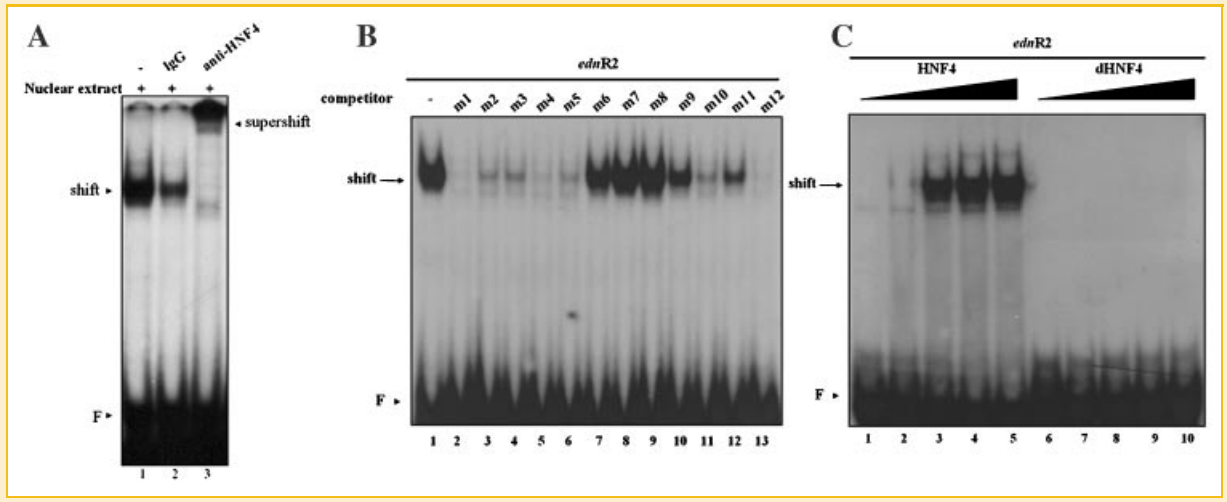

Fig. 4. Identification of the transcription factors recognizing the *edn*R2 segment. A: The labeled probes were incubated with HepG2 nuclear extract, and the DNA–protein complexes were separated by 6% non-denaturing acrylamide gel electrophoresis. Lanes 1, no antibody. Lanes 2 and 3 are in the presence of non-irrelevant antibody and anti-HNF4, respectively. B: The end-labeled *edn*R2 probe was incubated with 20 μg cell nuclear extract proteins from HepG2 cells in the absence of competitor (lane 1). Lanes 2–13 contained a 200-fold molar excess of unlabeled wild-type region 2 segment of *edn* (*edn*R2) and sequential two-base pair mutagenic *edn*R2 probe. The specific complex was indicated by the arrow on the left. EMSA was obtained from at least three independent experiments. C: The labeled *edn*R2 probes were incubated with recombinant HNF4. Lanes 1–5 contained serially increased amount of full-length HNF4 (0.375, 0.75, 1.5, 3, and 6 μg). Lanes 6–10 contained similar amount of HNF4 with the DNA-binding domain deleted (0.375, 0.75, 1.5, 3, and 6 μg). The specific complex was indicated by the arrow on the left. EMSA was obtained from at least three independent experiments.

the 9th–16th nucleotides "GTACTTTG" on *edn*R2 probe corresponding to the −342/−335 segment of the *edn* promoter were identified as the exact HNF4 binding site. This sequence is in consistence with the computational prediction by PROMO [Messeguer et al., 2002]. Subsequently, purified recombinant HNF4 and dHNF4, a mutant HNF4 lacking of the zinc fingers indispensable for the DNA-binding activity, were used in EMSA experiment. As increasing amounts (0.375, 0.75, 1.5, 3, and 6 μg) of recombinant HNF4-6H and dHNF4-6H were separately incubated with the *edn*R2 probe, Figure 4C showed that the degree of complex formation increased along with the ascending concentration used accordingly (Fig. 4C, lanes 2–5), whereas no shifted band was observed as dHNF4-6H was tested (Fig. 4C, lanes 7–10). Taken together, these results indicate that HNF4 may specifically recognize and bind to the $^{-342}$GTACTTTG^{-335} motif in *edn*R2 segment derived from the *edn* promoter.

HNF4 IS ASSOCIATED WITH *EDN* PROMOTER *IN VIVO*

ChIP assays were further conducted to examine whether HNF4 was associated with *edn* promoter in vivo. HepG2 cells were cross-linked with 1% formaldehyde, and the chromosomal DNA was sheared by repeated sonication. The DNA–protein complexes were immunoprecipitated using either rabbit IgG as a negative control or anti-HNF4 antibody. PCR primers were added to amplify the −385/−180 region of *edn*, and an evident amplification product was observed when anti-HNF4, rather than rabbit IgG was used for immunoprecipitation (Fig. 5). These results indicate that HNF4 indeed binds to human *edn* promoter in vivo.

HNF4 REGULATES *EDN* PROMOTER ACTIVITY

Knowing that HNF4 bound to *edn*R2 of *edn* promoter in vivo, whether HNF4 served as an activator or a repressor needed to be addressed. Thus, the effects of HNF4 in transactivation of *edn* promoter activity were examined. The reporter constructs were used to transfect HepG2 cells in the presence or absence of a HNF4 or DN-HNF4 expression vector. Taylor et al. [1996] identified DN-HNF4 as a selective dominant negative mutant, which forms defective heterodimers with wild-type HNF4, thereby preventing

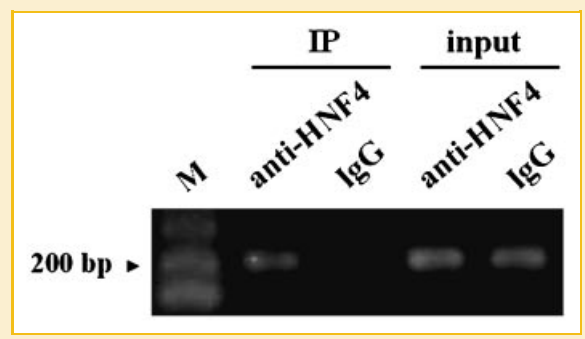

Fig. 5. In vivo binding of HNF4 to edn promoter. Chromatin immunoprecipitation (ChIP) assays were conducted to analyze the binding of proteins to the *edn* promoter. HepG2 were cross-linked with 1% formaldehyde. The Cross-linked DNA was immunoprecipitated with either rabbit IgG or antibodies to HNF4 (anti-HNF4). The ethidium bromide-stained PCR products were observed on a 2% agarose gel. PCR conditions were described in Materials and Methods Section. The ChIP assays were repeated three times with independent hepatocytes preparations.

DNA-binding and subsequent transcriptional activation by HNF4. Interestingly, the *edn* promoter activity was significantly inhibited in the presence of ectopically expressed HNF4, whereas that of pGL3-*edn*Δ (−81/−48) and *edn*-mutR2 was not affected. The inhibitory effect diminished when DN-HNF4 was introduced (Fig. 6A). As for controls, the persistence of over-expressed HNF4 and DN-HNF4 during the experimental time was confirmed in Figure 6B. Taken together, it was proved that HNF4 bound the *edn* promoter and repressed *edn* expression in HepG2 cells.

SP1 ASSOCIATES WITH HNF4 BUT NOT INVOLVES IN THE *EDN*R2 BINDING

It has been demonstrated that Sp1 acts as an activator of *edn* promoter through its binding to the 34-nt GC-rich segment [Wang et al., 2007]. Interestingly, HNF4 interaction with Sp1 was demonstrated [Kardassis et al., 2002]. Hence, whether HNF4 involve in Sp1/34-nt segment was investigated. The cell extract of HepG2 was separately subjected to immunoprecipitation using the anti-Sp1, anti-HNF4, or anti-IgG monoclonal antibodies. The immunoprecipitated complexes were analyzed by SDS–PAGE and Western blotting. As shown in Figure 7A, HNF4 was co-immunoprecipitated with endogenous Sp1 present in HepG2 cells, suggesting direct or indirect molecular association between these two factors. In order to investigate whether HNF4 could bind to *edn*R2 and interact with Sp1 simultaneously, DAPA was performed. The biotinylated DNA probe containing the *edn*R2 was synthesized, incubated with HepG2 nuclear extracts, and then coupled with

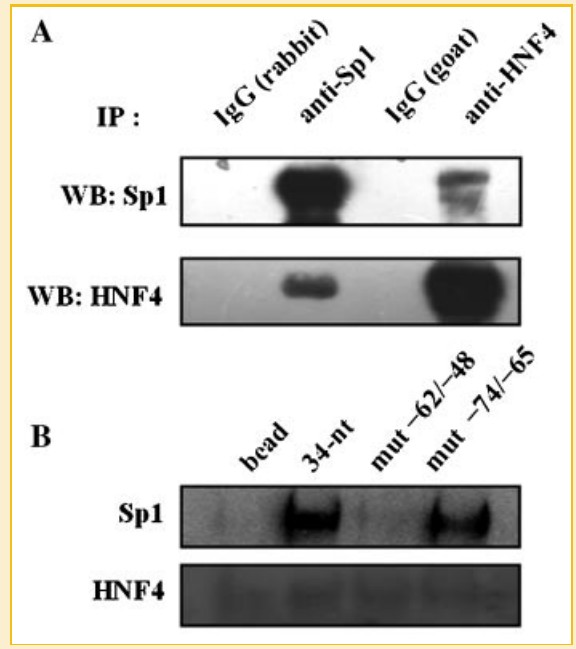

Fig. 7. Association of HNF4 with Sp1. A: HepG2 cell lysates were immunoprecipitated with IgG (rabbit), anti-Sp1, IgG (goat), and anti-HNF4 antibodies, resolved by 10% SDS–PAGE, transferred to membranes. B: Nuclear extracts were prepared from HepG2 cells and incubated with biotinylated oligonucleotide derived from the 34-nt of the *edn* promoter. The DAPA samples were separated by 10% SDS–PAGE and immunoblotted for Sp1 or HNF4.

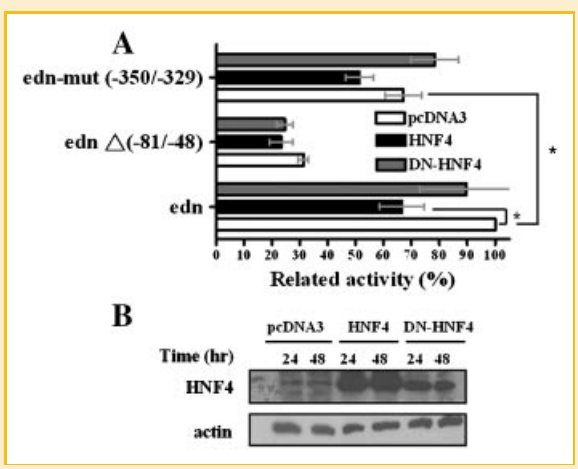

Fig. 6. Repression of *edn* promoter activity through HNF4 binding to the *edn*R2 of the *edn* promoter. A: Cotransfections and luciferase assays were performed with HepG2 cells in the presence of pcDNA3/HNF4 and pcDNA3/DN-HNF4. The average values of promoter activities were calculated as described in Materials and Methods Section and obtained from three independent experiments. $^{*}P < 0.05$ indicates versus control. B: To verify the persistence of HNF4 and DN-HNF4 during the experimental period, cells were transfected with equal amount of pcDNA3, pcDNA3/HNF4, or pcDNA3/DN-HNF4. Twenty-four and 48 h later, cells were harvested respectively, and protein lysates were separated by 12% SDS–PAGE, transferred to a PVDF membrane and probed for HNF4 and actin.

streptavidin-agarose beads. The DNA–protein complexes were resolved by 10% SDS–PAGE, followed by Western blot detection with anti-HNF4 or anti-Sp1 antibody. In Figure 7B, Sp1 was associated with the 34-nt segment but not with mutant 34-nt probe. Surprisingly, no HNF4 signal associated with the Sp1/34-nt complex was detected. Likewise, Sp1 was not detected along with the HNF4/*edn*R2 complex either (data not shown). To examine these unexpected results further, recombinant Sp1 and HNF4 were co-incubated overnight and subjected to immunoprecipitation with anti-HNF4 antibody. Figure 8A showed that anti-HNF4 antibody but not rabbit IgG pulled down recombinant HNF4. In addition, recombinant Sp1 was co-immunoprecipitated with recombinant HNF4. These results clearly indicate the direct interaction between Sp1 and HNF4, consistent with previous report [Kardassis et al., 2002]. To further investigate whether recombinant HNF4 affected binding activity of Sp1 to the 34-nt segment, recombinant Sp1 was incubated with the biotin labeled 34-nt probe and subjected to DAPA analysis. As shown in Figure 8B, Sp1 evidently bound to the 34-nt segment, whereas its DNA-binding ability was abolished in the presence of recombinant HNF4. Furthermore, the amount of MAZ binding to the 34-nt segment increased in the presence of HNF4 overexpression (Fig. 8C). This result suggests that excess HNF4 interacts with Sp1 and diminishes the DNA-binding of Sp1 to the 34-nt segment, which in turn leads to more MAZ binding to the 34-nt segment within *edn* promoter, and finally the repression of *edn* transcription is observed as shown in Figure 6A.

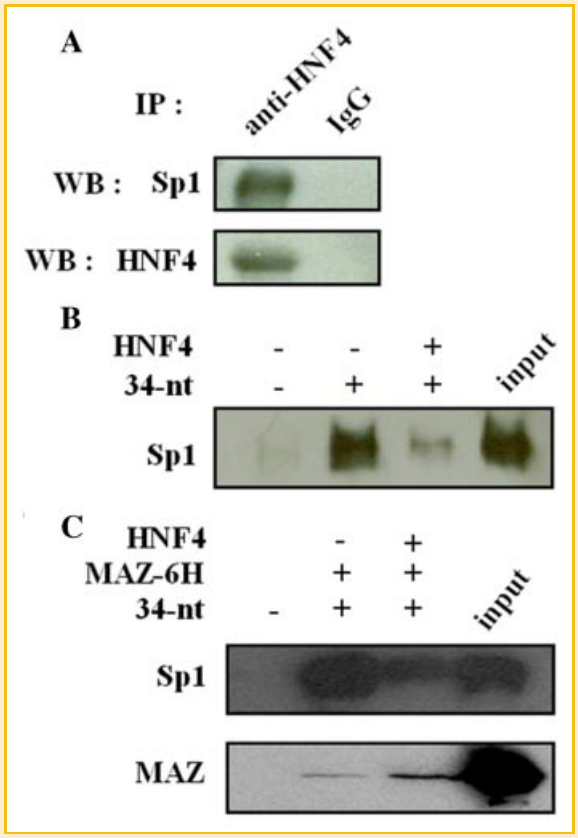

Fig. 8. Reduction of the DNA-binding activity of Sp1 to the 34-nt segment by HNF4-Sp1 interaction. A: The mixture of 100 ng of recombinant Sp1 and 1 μg of HNF4 was immunoiprecipitated and separated by 10% SDS-PAGE, transferred to membranes, and probed separately with anti-Sp1 and anti-HNF4 antibodies. B: Hundred nanograms of recombinant Sp1 and 5 μg of recombinant of HNF4 were separately incubated with biotinylated oligonucleotide derived from the 34-nt segment of the *edn* promoter. The DAPA samples were separated by 10% SDS-PAGE and immunoblotted for Sp1. C: HepG2 cells were overexpressed with MAZ-6H (pcDNA3/MAZ-6H) and then transfected with pcDNA3 or pcDNA3/HNF4. After 2 days incubation, cells were collected and the extracted nuclear-proteins were incubated with biotinylated oligonucleotides derived from the 34-nt segment of the *edn* promoter. The DAPA samples were separated by 10% SDS-PAGE and immunoblotted for Sp1 or His-tag. The amount of input was 20 μg nuclear extract.

OVEREXPRESSED HNF4 ENHANCES *EDN* PROMOTER ACTIVITY IN THE ABSENCE OF SP1

We have demonstrated that overexpressed HNF4 repressed *edn* promoter activity (Fig. 6), due to abolishment of DNA-binding activity of Sp1 (Fig. 8B). Thus, to elucidate whether HNF4 alone serves as an activator to enhance *edn* promoter activity, RNAi technique was applied to deplete Sp1 in the cell. Efficient depletion of Sp1 by the RNAi duplex was demonstrated [Wang et al., 2007]. When the RNAi against Sp1 was introduced into HepG2 cells, decreased *edn* promoter activity was observed. Interestingly, when we depleted Sp1 and then overexpressed HNF4, the *edn* promoter activity significantly increased (Fig. 9). It should be noted that the knockdown effect of HNF4 was not significant (data not shown), presumably due to low interference efficiency of the RNAi duplex.

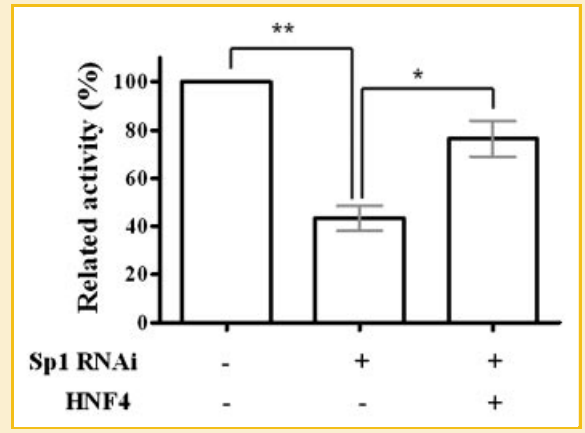

Fig. 9. Enhancement of *edn* promoter activity by HNF4 in the absence of Sp1. Luciferase assays using pGL3-basic containing *edn* promoter constructs and RNAi duplex against Sp1 were transfected into HepG2 cells in the presence or absence of HNF4 overexpression. The average values of promoter activities were calculated as described in Materials and Methods Section and obtained from three independent experiments. ** and * indicate $P < 0.001$ and $P < 0.05$ respectively versus control.

DISCUSSION

Sequence alignment analysis of the upstream 1 kb regions of human *edn* and *ecp* reveals 92% identity in their DNA sequences. However, the promoter activity of *edn* is significantly higher than that of *ecp* (Fig. 1A). Hence, it was hypothesized that sequence variations between human *edn* and *ecp* account for the discrepancy in regulation of promoter activity. In the swap experiment the replacement of *edn*R2 sequence motif with that of *ecp*R2 resulted in 40% reduction in promoter activity of the *edn* in HepG2 cells (Fig. 1B). The *edn*R2 segment is thus crucial for high promoter activity of *edn* in HepG2 cells.

EMSA revealed an evident retardant shift, suggesting that *edn*R2 contains a sequence for transcription factor binding in HepG2 cells. When nuclear extracts was incubated with the P^{32}-labeled oligonucletides correspondent to *edn*R2, HNF4 binding to the *edn*R2 probe was observed (Fig. 4). Direct binding between HNF4 and *edn*R2 in human *edn* promoter was further demonstrated by EMSA experiments in the presence of recombinant HNF4 (Fig. 4c). The HNF4 binding sequence in the *edn* promoter was identified employing EMSA scanning mutagenesis, and the results revealed that mutation of the "GTACTTTG" segment led to significant decrease in the *edn*R2 binding activity (Fig. 4b). The "GTACTTTG" responsive element for HNF4 in *edn*R2 corresponded to the −342/−335 position in the *edn* promoter. Interestingly, the HNF4 binding site characterized in *edn*R2 matched well with the HNF4 binding motif predicted by computational analysis [Pollak et al., 1996].

HNF4 is a transcription factor belonging to the member of the nuclear hormone receptor family. It forms a homodimer and binds DNA in liver, kidney, intestine, and pancreas cells. Although HNF4 is initially classified as an orphan receptor, its activity may be modulated by the binding of fatty acyl-CoA thioesters [Sladek et al., 1990; Hertz et al., 1998]. HNF4 acts as a positive transcriptional

regulator of many hepatocyte genes including *Fabl*, *Apob*, and *Cyp3a* [Watt et al., 2003]. Transcriptional regulation by HNF4 is accomplished by interactions with coactivation or corepresssion mediators (e.g., GRIP1, SRC-1, CBP/p300, PRMT) [Wang et al., 1998; Barrero and Malik, 2006]. The resulting HNF4/coactivator or HNF4/corepressor complex has intrinsic histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity, respectively [Wang et al., 1998; Soutoglou et al., 2001]. Histone modifications play an important role in the regulation of the accessibility of the DNA. They can promote an open chromatin structure, in which the DNA template is accessible for transcription factors, or facilitate chromatin condensation, leading to a transcriptionally non-permissive state [Heinzel et al., 1997; Nagy et al., 1997]. To investigate physical interaction between HNF4 and the *edn*R2 of the *edn* promoter in vivo, chromatin immunoprecipitation (ChIP) experiment was carried out and a PCR product containing the *edn*R2 segment was specifically pulled down by anti-HNF4, but not by anti-IgG antibody (Fig. 5), strongly indicating that HNF4 binds to *edn* promoter in vivo in HepG2 cells.

HNF4 and Sp1 have been reported to cooperatively regulate genes such as *apoCIII* [Kardassis et al., 2002] and *HO-1* [Takahashi et al., 2002]. Our previous study demonstrates that Sp1 is associated with the 34-nt sequence motif and acts as an activator during transcription of *edn* in HepG2 cells [Wang et al., 2007]. In this work, the direct interaction between HNF4 and Sp1 was also indicated (Fig. 7A). However, the association of HNF4 with Sp1/*edn*R2 complex or the association of Sp1 with HNF4/*edn*R2 complex was not observed (Fig. 7B). A possible explanation for this observation was that the overexpressed HNF4 associated with Sp1, which might block the interaction of Sp1 to its binding sequence within the 34-nt segment and resulted in the decrease of the *edn* promoter activity. Evidences that support this hypothesis are from our and other studies. Firstly, HNF4 devoid of DNA-binding region is unable to associate with Sp1 [Kardassis et al., 2002]. Secondly, Kardassis et al. [2002] proved that the Sp1-mediated transactivation was totally abolished by HNF4 overexpression in *Drosophila* SL2 cells. Thirdly, we have demonstrated that the presence of HNF4 abolishes the DNA-binding activity of Sp1 to the 34-nt segment (Fig. 8B), which in turn results in more MAZ binding to the 34-nt segment (Fig. 8C). Less Sp1 or more MAZ binding to the 34-nt segment has been shown to lead to the decrease of *edn* promoter activity [Wang et al., 2007]. Deletion of *edn*R2 alone also leads to the reduction of *edn* promoter activity (Fig. 1B). It is thus expected that HNF4 serves as an activator to enhance *edn* promoter activity. Indeed when we applied RNAi technique to eliminate the Sp1 effect, Overexpression of HNF4 enhanced *edn* promoter activity in the absence of Sp1 (Fig. 9). Therefore, we provided direct evidence to demonstrate the activating role of HNF4 in transcription regulation of *edn* promoter activity.

A simple model is proposed as followed. In this model, multiple transcription factors including HNF4 are recruited to the position −342/−335 on the human *edn* promoter to regulate *edn* transcription in HepG2 cells. In the presence of overexpressed HNF4, the excess free HNF4 can directly associate with Sp1 and abrogate DNA-binding activity of Sp1 to the 34-nt segment. Taken together, our studies reveal a complex regulatory mechanism related to human *edn* gene regulation.

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