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SHORT COMMUNICATION

Regulatory Element of the Hepatitis B Virus Core Promoter

VICTOR E. BUCKWOLD,[1] MIN CHEN,[1,2] and JING-HSIUNG OU[3]

Department of Molecular Microbiology and Immunology, University of Southern California, School of Medicine, Los Angeles, California 90033

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The negative regulatory element (NRE) of the hepatitis B virus (HBV) core promoter contains three subregions which act synergistically to suppress core promoter activity. One of these subregions, NREγ, is active in both HeLa cervical carcinoma cells and Huh7 hepatoma cells and was found to be bound by a protein factor present in both cell types. Here we show that the transcription factor RFX1 can bind to NREγ and transactivate the core promoter through this site. Mutations which abrogated the gene-suppressive activity of NREγ prevented RFX1 from binding to NREγ. In addition, RFX1 can bind simultaneously, most likely as a heterodimer, with the transcription factor MIBP1 to NREγ. In the absence of a cloned MIBP1 gene for further studies, we hypothesize that RFX1 acts with MIBP1 to negatively regulate the core promoter activity through the NREγ site. The ability of RFX1 to transactivate the core promoter raises the possibility that RFX1 may play a dual role in regulating HBV gene expression.

Hepatitis B virus (HBV) is a hepatotropic virus with a circular DNA genome of 3.2 kb. Despite its small size, the HBV genome encodes four genes which lead to the production of at least seven viral gene products. The expression of HBV genes is regulated by four promoters and two enhancer elements (for a review, see *1*). One of these promoters, termed the core promoter, regulates the transcription of HBV e antigen and core antigen mRNAs. The core mRNA also encodes the HBV DNA polymerase and is the pregenomic RNA required for replication of the HBV DNA genome.

The core promoter is preceded by an enhancer element termed ENII enhancer (Fig. 1). This enhancer, which has also been termed the core upstream regulatory element, activates the core promoter in a position- and orientation-dependent manner (*2*). An HNF4 binding site, which positively regulates the core promoter activity, has been identified in the ENII enhancer (*3*). In cotransfection experiments, HNF4 has been found to transactivate the core promoter 10- to 20-fold in the HeLa cervical carcinoma cell line. Interestingly, this transactivation of the core promoter activity is suppressed by an upstream negative regulatory element (NRE) in an orientation-independent manner in HeLa cells (*3–5*).

[1] These authors contributed equally to this work.

[2] Current address: National Institutes of Health, NIAMS, Arthritis Rheumatology Branch, Lymphocyte Cell Biology Section, Building 10, 9N262, 9000 Rockville Pike, Bethesda, MD 20892.

[3] To whom correspondence and reprint requests should be addressed.

By performing deletion-mapping analysis, the NRE has been divided into three subregions termed NREα, NREβ, and NREγ (*4*). In HeLa cells, each of these subregions possesses a weak 2- to 3-fold suppressing activity, but together they generate a strong synergistic suppressing effect of 10- to 20-fold. In liver-derived Huh7 hepatoma cells, only the NREγ subregion is active; the NREα and NREβ subregions are not (*4, 6*). The active NREγ motif has been mapped to a 20-basepair region between nucleotides 1605 and 1625 by DNase I footprinting, methylation interference, and mutagenesis analyses. In a UV cross-linking experiment, a ubiquitous protein factor approximately 130 kDa in size was found to bind to the NREγ site (*4*).

As shown in Fig. 1, there is an extensive sequence homology between the NREγ motif and the consensus RFX1 transcription factor binding site (*7*). RFX1 is approximately 130 kDa in size and was initially isolated due to its ability to bind to the X-box motif of HLA class II promoters (*8*). This protein factor, which was subsequently found to be identical to the methylation-dependent DNA-binding protein (MDBP) (*9*), can function as a transactivator of the HBV ENI enhancer (*10*). Due to the sequence homology between the NREγ motif and the RFX1-binding site, and to the size similarity between the 130-kDa NREγ-binding factor and RFX1, we have examined whether these two factors might be identical. We performed an electrophoretic mobility shift assay (EMSA) using a double-stranded oligonucleotide containing the NREγ sequence as the probe. As shown in Fig. 2A, in

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the presence of the HeLa nuclear extract, a shifted band (denoted by an arrowhead) was detected (lane 2), indicating binding by a protein factor to the NREγ sequence. This shifted band could be entirely supershifted by a rabbit antibody directed against RFX1 (lane 3), but not by a control rabbit antibody (lane 4). A similar result was obtained when Huh7 nuclear extract was used for the experiment (data not shown). Therefore, RFX1 appears to bind NREγ. To confirm this result, a different approach employing both EMSA and Western blot assay was used to investigate whether RFX1 could bind to the NREγ site. HeLa nuclear extract incubated with or without unlabeled NREγ oligonucleotide was subjected to electrophoresis in a low-ionic-strength gel, transferred to nitrocellulose membrane, and probed with anti-RFX1 antibody. As shown in Fig. 2B, an intense signal was detected only when the NREγ oligonucleotide was used in the binding reaction (lane 2, denoted by an arrow). The lack of the Western blot signal in the absence of the NREγ oligonucleotide was likely due to the inability of the RFX1 protein to enter the nondenaturing, low-ionic-strength gel during electrophoresis (*11*). This result supports the result of Fig. 2A and indicates that RFX1 indeed binds to the NREγ sequence.

To examine the effect of RFX1 on NREγ activity, we next performed transfection experiments in Huh7 hepatoma cells. As a control, a plasmid containing mutations in the NREγ site was also used. To ensure that the mutations introduced into the NREγ site would prevent binding by the NREγ factor, we first performed an EMSA experiment. As shown in Fig. 3A, while the wild-type oligonucleotide could be bound by the NREγ factor (lane 2), the mutant could no longer be bound by the protein factor (lane 4). The full-length NRE, with or without the mutations in the NREγ site, and its downstream core promoter were next linked to the chloramphenicol acetyl transferase (CAT) reporter and cotransfected with an RFX1 expression plasmid into Huh7 cells. As shown in Fig. 3B, cotransfection with RFX1 led to an approximately fourfold increase of the core promoter activity of pUCAT1 which contained the wild-type NREγ sequence. In contrast, pUCAT10, which contained the mutated NREγ sequence, was no longer responsive to transactivation by RFX1. These results indicate that the transactivation of the core promoter by RFX1 is mediated by the NREγ site and that the NREγ protein-binding activity is essential for this transactivation. Similar transactivation results were obtained with HeLa cells in our preliminary cotransfection experiments.

The finding that RFX1 stimulated core promoter activity was surprising, because if RFX1 is indeed the NREγ

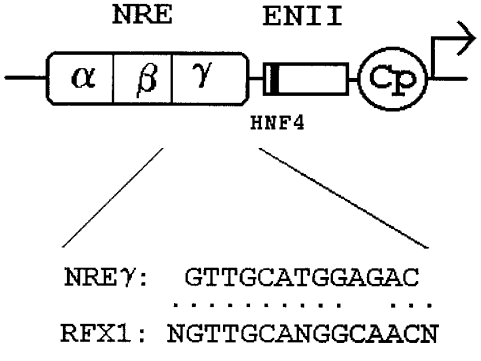

FIG. 1. HBV core promoter and sequence homology between NREγ and RFX1. α, β, and γ represent the three subregions of the NRE. The solid box represents the HNF4 binding site. Cp, core promoter; ENII, the ENII enhancer. Dots mark the identical nucleotide residues between the NREγ motif and the consensus RFX1-binding site (*7*). The arrow marks the initiation sites of the C gene transcripts.

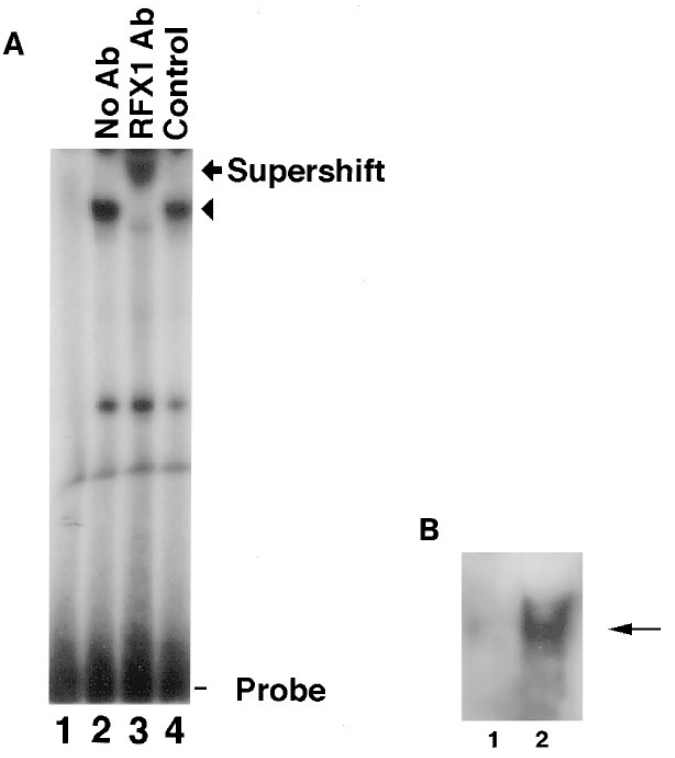

FIG. 2. Analysis of binding by RFX1 to the NREγ motif. (A) Supershift assay. EMSA was performed as previously using 5 μg HeLa nuclear extract and a 28mer double-stranded oligonucleotide containing the NREγ-binding site (*4*). Lane 1, probe alone; lane 2, with nuclear extract; lane 3, nuclear extract plus rabbit anti-RFX1 antibody (gift of M. Peterlin and N. Jabrine-Ferrat); lane 4, nuclear extract plus a control antibody. The NREγ-specific band and the supershifted band are denoted by an arrowhead and an arrow, respectively. The position of free probe is also marked. (B) EMSA–Western blot assay. The procedures of this assay have been described in detail in (*11*). Briefly, EMSA was performed as in (A) except that the NREγ oligonucleotide was not labeled, the amount of the oligonucleotide used was increased 10-fold, and the thickness of the gel was increased from 0.8 to 1.5 mm. Following electrophoresis, the gel was transferred to nitrocellulose membrane in 0.4% sodium dodecyl sulfate, 48 m*M* Tris base, 39 m*M* glycine, and 20% methanol. The membrane was then hybridized with anti-RFX1 primary antibody and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma Biochemicals). The signal was developed using a Chemiluminescence kit (Dupont-NEN). Lane 1, without NREγ DNA; lane 2 with NREγ DNA. The arrow marks the location of the DNA–RFX1 complex.

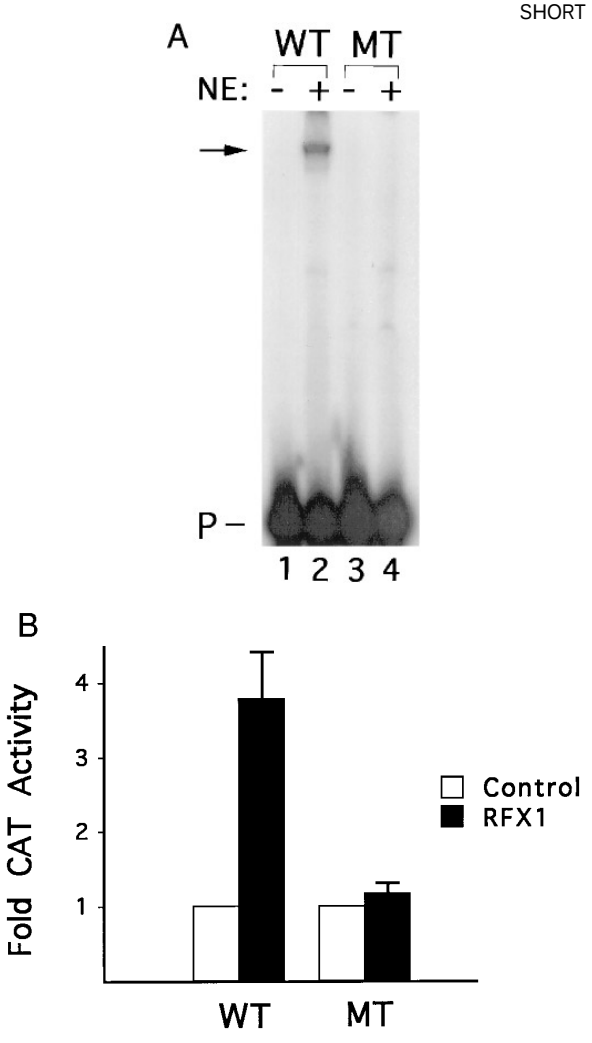

FIG. 3. (A) Analysis of NREγ factor binding to the mutated NREγ sequence. The mutated double-stranded NREγ oligonucleotide used in EMSA was

5′ TCGAGCACTGATATCCTGCAGCACCGTG 3′
3′ CGTGACTATAGGACGTCGTGGCACAGCT 5′.

In this mutated sequence, 12 basepairs (underlined) of the NREγ motif were replaced with other nucleotides. Lanes 1 and 2, NREγ wild-type (WT) probe employed; lanes 3 and 4, NREγ mutant (MT) probe; lanes 1 and 3, without (−) nuclear extract (NE); lanes 2 and 4, with (+) nuclear extract. No shifted signal (denoted by an arrow) was detected when the mutated NREγ probe was used for EMSA. The position of free probe (P) is also indicated. (B) Transactivation of the core promoter by RFX1. 60-mm plates of Huh7 cells were transfected using the $CaPO_4$ precipitation method (*16*) with 4 μg wild-type NREγ motif-containing pUCAT1 (WT) or the mutated NREγ motif-containing pUCAT10 (MT), and 5 μg of either pRc/CMV (Invitrogen) control or the RFX1 expression plasmid, pCMV-RFX1. Two days posttransfection, cell lysates were analyzed for CAT activity as previously (*4*). The results shown represent the mean and the standard deviation of three different transfection experiments. Fold CAT activity is relative to the pRc/CMV control without pCMV-RFX1 cotransfection. The construction of pUCAT1 has been described (*4*). pUCAT10 is identical to pUCAT1 except that it contains the nucleotide mutations shown above. pCMV-RFX1 was constructed by inserting the *Eco*RI fragment of pUC19-RFX1 (gift of M. Peterlin and N. Jabrine-Ferrat) containing the RFX1 coding sequence into the *Eco*RI site of a CMV vector (*17*). In this plasmid, the expression of RFX1 was under the control of the cytomegalovirus immediate early promoter. In the absence of RFX1, the activity of MT is two- to threefold higher than that of WT. This result is consistent with our previous findings (*4*).

factor, it is anticipated to suppress, rather than to stimulate, core promoter activity. Recently, it has been demonstrated that RFX1 can form a heterodimer with the myc intron-binding protein (MIBP1) and that this heterodimer possesses a gene-suppressive activity (*7*). For this reason, we have investigated whether the NREγ factor is a heterodimer of RFX1 and MIBP1 by performing a supershift assay using an antibody directed against MIBP1. As shown in Fig. 4, the anti-MIBP1 antibody, which does not cross-react with RFX1 (*7*), supershifted all of the NREγ-binding activity (lane 2) while a control serum did not (lane 3). Since the anti-RFX1 antibody also entirely supershifted the NREγ-binding activity (Fig. 2A), RFX1 and MIBP1 must bind simultaneously, most likely as a heterodimer, to the NREγ sequence. The ubiquitous nature of RFX1 and MIBP1 is consistent with the observation that the NREγ factor is present in both Huh7 and HeLa cells. In contrast to RFX1, the role of MIBP1 in the regulation of the NREγ activity cannot be assessed at present as the gene encoding this protein factor has not yet been isolated.

While our EMSA results indicate that RFX1 and MIBP1 can bind simultaneously to the NREγ site, our previous cross-linking experiment indicated that only a 130-kDa protein bound to the NREγ DNA (*4*). Since RFX1 and MIBP1 are approximately 130 and 160 kDa in size (*7*),

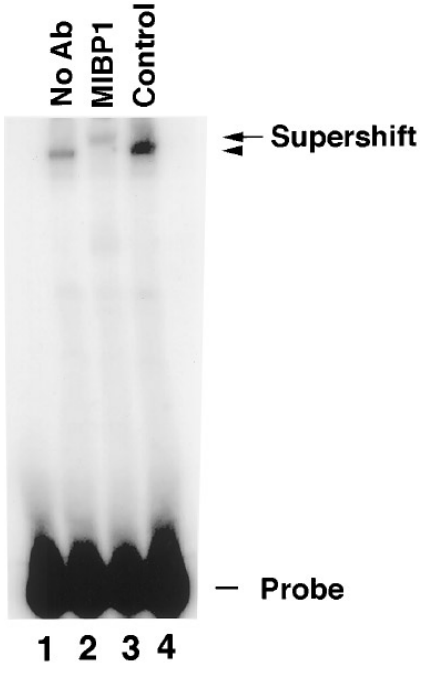

FIG. 4. Supershift analysis of the NREγ factor with anti-MIBP1 antibody. The supershift experiment was performed as in Fig. 2A except an anti-MIBP1 antibody (gift of M. Zajac-Kaye) was used. Lane 1, probe alone; lane 2, with HeLa nuclear extract; lane 3, with nuclear extract and anti-MIBP1 antibody; lane 4, with nuclear extract and preimmune rabbit serum. The arrow indicates the supershifted band while the arrowhead indicates the NREγ-specific bandshift. The position of free probe is also indicated.

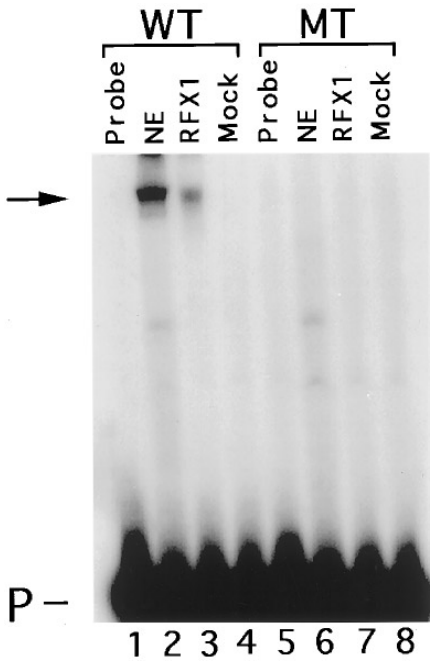

FIG. 5. EMSA assay with *in vitro* synthesized RFX1. Lanes 1–4, the wild-type (WT) NREγ probe; lanes 5–8 the mutated (MT) NREγ probe. Lanes 1 and 5, free probe; lanes 2 and 6, HeLa nuclear extract added; lanes 3 and 7, 1 μl RFX1 synthesized using rabbit reticulocyte lysate (Promega); lanes 4 and 8, 1 μl control reticulocyte lysate. The RNA used for RFX1 synthesis was from the plasmid pGEM-RFX1 (gift of M. Peterlin and N. Jabrine-Ferrat) which was linearized with *Hin*dIII followed by RNA synthesis using SP6 RNA polymerase (Promega). The arrow indicates the site of shifted bands.

respectively, it would appear that only RFX1 was cross-linked to the NREγ DNA. If this is indeed the case, it would imply that only RFX1 binds directly to the NREγ site and MIBP1 binds to the same site indirectly through its interaction with RFX1. The ability of RFX1 to bind to NREγ in the absence of MIBP1 is demonstrated in Fig. 5. As shown in the figure, RFX1 synthesized *in vitro* could bind by itself to the NREγ probe but not to the mutated NREγ probe. The experiment to test whether MIBP1 can also bind directly to NREγ must await the isolation of this gene.

Our finding is reminiscent of that of the EP (EF-C) element of the HBV ENI enhancer (*7, 12*), the MIF-1 element of the myc gene intron I (*7*), and the major histocompatibility complex II RFX1 binding sites (*7*). In those cases, RFX1 was found to interact with MIBP1 when bound to these elements. Furthermore, both multimerized EP and MIF-1 elements could serve as cell type-specific silencers. Similar to our finding, in cotransfection experiments, RFX1 was found to stimulate gene expression through the EP site (*13*). Since the HBV ENI enhancer requires the EP site and its juxtaposed HNF3- and HNF4-binding sites for basal activity (*3, 4, 14–16*), it has been postulated that these *cis*-acting elements play an important role converting the silencer activity of the EP site into an enhancer (*13*). In the case of NREγ, it is possible that flanking *cis*-acting elements also play important roles in modulating RFX1 activity to regulate core gene expression. Alternatively, it is also possible that RFX1 functions as a positive regulator of the core promoter, and, in the presence of MIBP1, which can bind to RFX1 (*7*), RFX1 is converted into a negative regulator to suppress the core promoter through its NREγ-binding site. This dual activity of RFX1 may be important for differential regulation of HBV gene expression during the viral life cycle.

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