JOURNAL OF VIROLOGY, Mar. 1994, p. 1758–1764
0022-538X/94/$04.00+0

Vol. 68, No. 3

Repression of Enhancer II Activity by a Negative Regulatory Element in the Hepatitis B Virus Genome

WEI-YU LO AND LING-PAI TING*

Graduate Institute of Microbiology and Immunology, National Yang-Ming Medical College, Shih-Pai, Taipei 11221, Taiwan, Republic of China

Received 13 September 1993/Accepted 29 November 1993

Enhancer II of human hepatitis B virus has dual functions in vivo. Located at nucleotides (nt) 1646 to 1741, it can stimulate the surface and X promoters from a downstream position. Moreover, the same sequence can also function as upstream regulatory element that activates the core promoter in a position- and orientation-dependent manner. In this study, we report the identification and characterization of a negative regulatory element (NRE) upstream of enhancer II (nt 1613 to 1636) which can repress both the enhancer and upstream stimulatory function of the enhancer II sequence in differentiated liver cells. This NRE has marginal inhibitory effect by itself but a strong repressive function in the presence of a functional enhancer II. Mutational analysis reveals that sequence from nt 1616 to 1621 is required for repression of enhancer activity by the NRE. Gel shift analysis reveals that this negative regulatory region can be recognized by a specific protein factor(s) present at the 0.4 M NaCl fraction of HepG2 nuclear extracts. The discovery of the NRE indicates that HBV gene transcription is controlled by combined efforts of both positive and negative regulation. It also provides a unique system with which to study the mechanism of negative regulation of gene expression.

Hepatitis B virus (HBV) infection is one of the world's leading health problems. Chronic carriers of HBV have a greatly elevated risk of developing cirrhosis and hepatocellular carcinoma (1, 21). HBV is one of the smallest DNA viruses; it has a partially double-stranded 3.2-kb viral genome. There are four open reading frames, which code for the surface, core, polymerase, and X protein (2, 5, 22). The transcription of these genes is under the control of four promoters, two for surface (SPI and SPII), one for core and polymerase (CP), and the other for X (XP) (3, 4, 8, 16, 24, 29, 30). Two enhancers, enhancer I and enhancer II, have been identified so far in the HBV genome. These enhancers have been shown to stimulate the viral promoters in a hepatocyte-specific manner, which may explain in part the hepatotropism of this virus (8, 20, 23, 26, 27).

We have previously reported the characterization of enhancer II. Located downstream of enhancer I and within the X open reading frame, this enhancer is composed of two interacting sequence motifs, a 23-bp box α (nucleotides [nt] 1646 to 1668) and a 12-bp box β (nt 1704 to 1715). Cooperation of the two elements is required for enhancer function. This enhancer can stimulate the transcriptional activities of SPI, SPII, and XP in a position- and orientation-independent manner (25–27).

Interestingly, both box α and box β are also constituents of the core upstream regulatory sequence (CURS) and can positively regulate the transcription of the downstream basal core promoter (BCP) individually. Box α and box β can strongly and moderately, respectively, stimulate the activity of BCP in only a position- and orientation-dependent fashion (24). Although the *trans*-acting factor(s) that mediates these functions is not yet fully understood, C/EBP-like protein(s) and HNF-4 have been shown to be likely candidates (9, 16, 26).

In this paper we report the identification of a negative regulatory element (NRE) upstream of box α that abolishes the upstream stimulatory function of the CURS on the BCP. In addition, the NRE can dominantly repress the enhancer activity of enhancer II in differentiated liver cells. This latter repressive function appears to be dependent on enhancer II in that the repressive effect is seen mainly in the presence of an intact enhancer II. Fine mapping by linker-scanning analysis reveals that the sequence between nt 1616 to 1621 is required for the repressive effect on enhancer II.

* Corresponding author. Mailing address: Graduate Institute of Microbiology and Immunology, National Yang-Ming Medical College, Shih-Pai, Taipei 11221, Taiwan. Phone: 886-2-8222400. Fax: 886-2-8212880.

MATERIALS AND METHODS

Plasmid construction and preparation. The HBV sequence used in this study is of the *adw* subtype. Numbering of the HBV sequence begins at the unique *Eco*RI site, which is nt 1. All reporter plasmids used in transfection experiments contain a head-to-tail trimeric tandem repeat, referred to as A3, of a 237-bp *Bcl*I-*Bam*HI fragment from the simian virus 40 (SV40) polyadenylation signal. A3 is placed 5′ of sequences assayed for promoter activity and has been shown to stop transcription readthrough from spurious upstream initiation. The parental plasmid containing A3 on a pGEM backbone is designated as pA3/RIdB.

pHBV3.8 was constructed by three-way ligation. The *Eco*RI-*Fsp*I fragment containing the A3 and core promoter sequence (nt 1402 to 1804) was taken from pA3(1402–1851)CAT. This fragment was ligated to the *Fsp*I-*Pst*I fragment from HBV (nt 1805 to 25) and an *Eco*RI-*Pst*I-restricted pGEM vector backbone. The resulting plasmid was further extended with the *Pst*I-fragment containing the HBV sequence from nt 25 to 1990 isolated from pSpHBs1775 at the unique *Pst*I site to create pHBV3.8.

Plasmids pHBV3.65, pA3(1402–1851)CAT, pA3(1636–1851)CAT, and pA3(1744–1851)CAT were described previously (24). Plasmid pA3(1613–1851)CAT was constructed by replacing the sequence from nt 1402 to 1851 of pA3(1402–1851)CAT with the 239-bp *Nla*III-*Rsa*I fragment (nt 1613 to 1851) of the HBV sequence.

Plasmid pA3SVpCAT contains the bacterial chloramphenicol acetyltransferase (CAT) gene driven by the SV40 early

promoter. The 129-bp *Pst*I fragment containing the HBV sequence from nt 1613 to 1741, derived from p(1613–1741)/3Zf(+), was subcloned into the *Pst*I site immediately downstream of the polyadenylation site in pA3SVpCAT. The same 129-bp *Bam*HI fragment eluted from p(1613–1741)/3Zf(+) was inserted into the *Bam*HI site upstream to the promoter in pA3SVpCAT. The resulting plasmids which carry insertions at downstream or upstream positions in either orientation were obtained and designated as pA3SVpCAT(1613–1741), pA3SVpCAT(1741–1613), pA3(1613–1741)SVpCAT, and pA3(1741–1613)SVpCAT, respectively. The set of plasmids shown in Fig. 4 was constructed by insertion of the synthetic oligonucleotides

```
        1613                    1636
          ●                       ●
5'-gatctGAGACCACCCTGAACGCCCATCAGg-3'
    3'-aCTCTGGTGGCACTTGCGGGTAGTCcctag-5'
```

(coding strand on top; the *Bam*HI-*Bgl*II linker sequence is shown in lowercase letters) into the *Bam*HI site located upstream of the promoter or downstream of the polyadenylation site of pA3SVpCAT, respectively.

The CAT fragments of pA3(1613–1636)SVpCAT and pA3(1636–1613)SVpCAT were replaced with the CAT(1636–1741) fragments to generate pA3(1613–1636)SVpCAT(1636–1741) and pA3(1636–1613)SVpCAT(1636–1741) shown in Fig. 7.

The synthetic oligonucleotides corresponding to the wild-type and mutant sequences of HBV from 1613 to 1668 (see Fig. 6) were annealed and inserted into the *Bam*HI site downstream of pA3SVpCAT to generate pA3SVpCAT(1613–1668WT), pA3SVpCAT(1613–1668AB), or pA3SVpCAT (1613–1668MT). The 1.9-kb *Sac*I-*Xba*I fragments from plasmids SVpCAT(1613–1668WT), SVpCAT(1613–1668AB), and SVpCAT(1613–1668MT) were first blunt ended with Klenow fill-in and used to replace the *Sac*I-*Stu*I segment in pA3SVpCAT(1636–1741) to generate pA3SVpCAT(1613–1668)WT+IIB and its mutant derivatives.

The fragment containing A3(−95)SPICAT (a CAT reporter gene unit driven by the SPI promoter of HBV corresponding to nt 2717 to 2828) was inserted upstream of p(1613–1741)/3Zf(+) and p(1636–1741)/3Zf(+) to generate pA3(−95)SPICAT(1613–1741) and pA3(−95)SPICAT(1636–1741), respectively. The promoter sequences of pA3SVpCAT(1613–1741) and pA3SVpCAT(1636–1741) were replaced by the X promoter sequence (from nt 1116 to 1374) to create pA3XpCAT(1613–1741) and pA3XpCAT(1636–1741), respectively.

Cell lines, transfection, and CAT assay. The human hepatoma cell line HepG2 and HuH-7 were cultured in Dulbecco modified Eagle medium (Flow Laboratories, North Ryde, Australia) supplemented with 10% fetal calf serum, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, 1% nonessential amino acids, and 25 mg of amphotericin B (Fungizone) per ml at 37°C in a 5% CO_2 atmosphere. Cells were transfected with plasmids that had been through double bandings on CsCl gradients by the calcium phosphate precipitation method (7).

CAT assays were performed by the method of Gorman et al. (6) with previously described modifications (3). The CAT activity was normalized against the CAT activity exhibited by a control plasmid, pA3SV2CAT, which was taken as 100%. In pA3SV2CAT, the expression of the CAT gene is driven by the SV40 early promoter and 72-bp enhancer. When the CAT activity was high, assays were performed on serially diluted cell lysates to ensure that all assays were done in the linear range of CAT activity.

Assay for endogenous DNA polymerase activity. To assay for endogenous DNA polymerase activity, the culture supernatant was collected 3 days after transient transfection, treated with 1% Nonidet P-40 for 1 h at room temperature, and centrifuged at 17,000 × *g* for 30 min at 4°C. The supernatant was collected and recentrifuged at 225,000 × *g* for 1 h at 4°C. The pellet from the second centrifugation, which contains HBV viral core particles, was resuspended in TNE buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 0.1 mM EDTA) and assayed for endogenous polymerase activity as previously described (24).

Preparation and heparin-Sepharose fractionation of nuclear extracts. Nuclear extracts from the differentiated human hepatoma cell line HepG2 were prepared as previously described (3). The extracts were fractionated at 4°C as previously described (26). The crude and fractionation nuclear extracts were aliquoted, quickly frozen under liquid nitrogen, and kept frozen at −70°C.

Gel shift analysis. The probe was prepared with annealed double-stranded oligonucleotides (200 ng) corresponding to the NRE sequence of HBV (as shown in plasmid constructions) and end labeled via fill-in with [α-^{32}P]dATP (3,000 Ci/mmol; Amersham Corp., Amersham, England) at either terminus of a *Bgl*II-*Bam*HI linker site.

Nuclear extracts (10 μg) were incubated in a 20-μl reaction mixture containing 1 μg of poly(dI-dC)(dI-dC) (Pharmacia, Inc.), 10× binding buffer (170 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid HEPES [pH 7.9], 20% glycerol, 4.2 mM EDTA, 3 mM dithiothreitol, 100 mM KCl, 62.5 mM $MgCl_2$), 12.5% glycerol, and 2.2 × 10^5 cpm of labeled DNA. Reaction mixture were incubated at 30°C for 30 min, and protein-DNA complexes were resolved on 4% polyacrylamide (acrylamide/bisacrylamide weight ratio, 30:1) made in 1× TBE (90 mM Tris-borate [pH 8.0], 2 mM EDTA). Electrophoresis was performed at 150 V for 2.5 h at 4°C. The gel was dried and autoradiographed. For competition experiments, 25- and 250-fold molar excesses of unlabeled double-stranded oligonucleotides were preincubated with nuclear extracts on ice for 5 min before addition of labeled probe.

RESULTS

Functional identification of an NRE located upstream of enhancer II. The core promoter of HBV can be functionally divided into two regions, the BCP, which can direct the precise initiation of the precore and pregenomic RNAs, and the CURS, which stimulates the core promoter activity (24). To test whether sequences further 5′ have any modulatory effect on the core promoter, we made two HBV constructs, pHBV3.8 (nt 1402 to 1990) and pHBV3.65 (nt 1636 to 1990). Both contain more than a unit length of HBV genome and differ in the amount of HBV sequence upstream of the core promoter. When these constructs were transiently transfected into a differentiated human hepatoma cell line, HuH-7, various numbers of 42-nm virions and 27-nm core particles can be made that are determined by the strength of their respective core promoters. As shown in Fig. 1, pHBV3.8 can lead to the production of a much smaller number of virions and core particles than pHBV3.65 does. This observation indicates that the HBV sequence from 1402 to 1635 may contain a negative regulatory element(s) which functions predominantly in a position-dependent manner (i.e., upstream position) since the same sequence is repeated once toward the 3′ ends of all HBV constructs.

To further localize this negative element(s), different lengths of the core promoter sequence from nt 1402 to 1851, nt 1613 to 1851, and nt 1636 to 1851 were placed in front of a

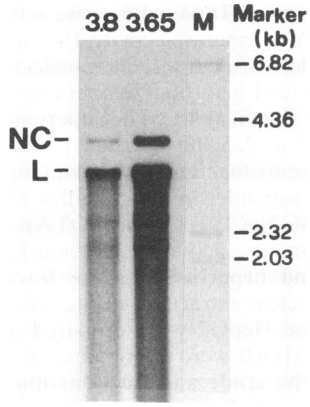

FIG. 1. Effect of the HBV sequence from nt 1401 to 1635 on the HBV virion and core particle production in transient-transfection experiments. Plasmids pHBV3.8 and pHBV3.65, each containing more than the unit length of HBV viral DNA and referred to as 3.8 and 3.65, respectively, were tested in these experiments. Plasmid pHBV3.8 contains sequence from nt 1401 through 1990, and pHBV3.65 contains nt 1636 through 1990. These plasmids were transfected into HuH-7 cells by the calcium phosphate precipitation method, and media from the transfectants were collected 3 days later to assay for the production of HBV virions and core particles. The amounts of virions and core particles produced were quantified by the DNA repairing assay. L and NC represent linear and nicked circular forms, respectively, of HBV DNAs. The molecular size marker is in lane M. A pA3SV2CAT vector, which contains a CAT gene driven by the SV40 early promoter and 72-bp enhancer, was included in all transfections as an internal control for transfection efficiency.

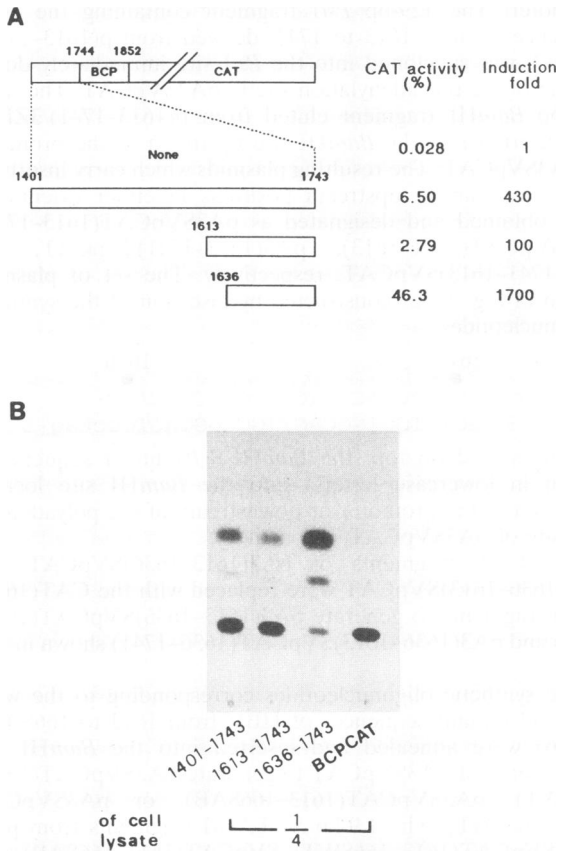

FIG. 2. Presence of an NRE upstream of the CURS. HBV sequences that contain the BCP and decremental amounts of the 5′ sequences were inserted upstream of a promoterless CAT reporter gene. These plasmids were transfected into HepG2 cells by the calcium phosphate precipitation method, and CAT assays were performed on cell lysates harvested 2 days after transfection. (A) Schematic representation of the plasmid constructs; the CAT activities they exhibited, normalized against that of pA3SV2CAT, which was included in all experiments as a control for transfection efficiency; and the fold induction. Each cell lysate was serially diluted to allow a quantitative measurement of the CAT activities. The values presented here are the average of five independent experiments with a standard deviation of 5%. (B) Representative autoradiogram of a CAT assay.

promoterless CAT construct and assayed for their transcriptional activities (Fig. 2). These plasmids were transiently transfected into the differentiated human hepatoma cell line HepG2, and CAT assays were performed on cell lysates collected 2 days later. Compared with the CAT activity produced by HBV sequence from nt 1636 to 1851, an addition of the sequence from nt 1613 to 1635 results in a 17-fold reduction of the CAT activity whereas a further inclusion of the sequence from nt 1402 to 1612 increases the CAT activity by 4.3-fold. These results localize the presence of one negative element to the HBV sequence from nt 1613 to 1635 and suggest the presence of another positive element(s) or combined positive and negative elements in the upstream sequence. A similar repressive effect of the sequence from nt 1613 to 1635 was observed in the HuH-7 cell line (data not shown).

Since the CURS coincides with enhancer II (24), it is important to understand whether the sequence from nt 1613 to 1635 can also negatively regulate enhancer II activity. This possibility was tested by inserting the HBV sequences from nt 1613 to 1741 and from nt 1636 to 1741 downstream of a CAT reporter gene driven by the SV40 early promoter and comparing their effects on the promoter activities (Fig. 3). As previously shown (25), enhancer II, which contains the sequence from nt 1636 to 1741, can stimulate the SV40 early promoter 28-fold in HepG2 cells. The sequence from nt 1613 to 1741, however, fails to stimulate the activity of the SV40 early promoter. The same result was also observed in HuH-7 cells (data not shown). This result indicates that the addition of the sequence from nt 1613 to 1635 has seemingly led to the repression of the second enhancer of the HBV. The sequence from nt 1613 to 1635 is therefore referred to as the negative regulatory element (NRE) of enhancer II.

It was shown previously that the enhancer II (nt 1636 to 1741) can activate the SV40 early promoter in a position- and orientation-independent manner (25). The NRE plus enhancer II sequence (from nt 1613 to 1741), however, fails to exhibit significant enhancer activity. In other words, NRE appears to completely efface the enhancer II activity. This repressive effect of the NRE is seen when the NRE plus enhancer II sequence is placed in either a sense or antisense orientation and either upstream or downstream of a CAT reporter gene driven by the SV40 early promoter (data not shown). The NRE appears to be functional in an orientation- and position-independent manner with respect to a test promoter—the SV40 early promoter.

Dependence of NRE function on a functional enhancer II. It is possible that the negative regulatory effect of NRE which is contained within the sequence immediately upstream of enhancer II results from its direct repression of the SV40 early promoter. This likelihood was tested by placing the NRE sequence (from nt 1613 to 1635), in both orientations, either upstream or downstream of the CAT reporter gene on a pA3SVpCAT plasmid. As shown in Fig. 4, the NRE sequence

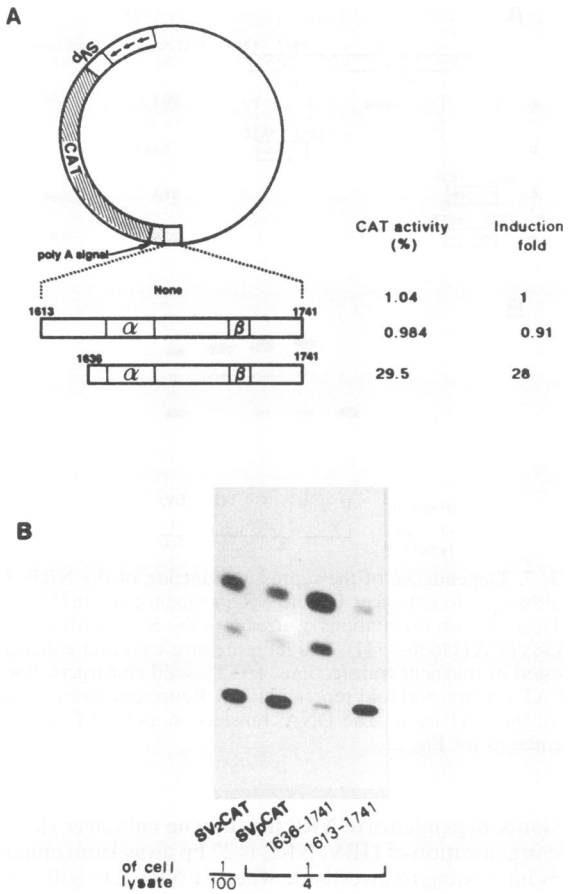

FIG. 3. Abolition of the stimulatory effect of enhancer II by the NRE. The HBV sequences from nt 1613 to 1741 and from nt 1636 to 1741 were inserted downstream from a CAT reporter gene driven by an SV40 early promoter without the 72-bp enhancer. The transfection and CAT assay were performed as described in the legend to Fig. 2. (A) Plasmid constructs, their normalized CAT activities, and fold induction. (B) Representative autoradiogram of the CAT assay.

can decrease the transcriptional activity by only threefold at any position. Similarly, only a twofold repressive effect is seen when the sequence downstream of NRE is also included (nt 1613 to 1668 [Fig. 5B, lane d]). Taken together, these results

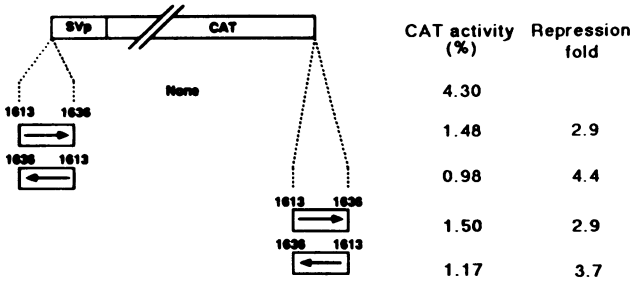

FIG. 4. Effect of NRE on the SV40 early promoter. The NRE sequence (nt 1613 to 1636) was placed in both orientations and either upstream or downstream of a CAT reporter gene driven by an SV40 early promoter. The plasmid constructs, normalized CAT activities, and fold induction are shown. The DNA transfection and the CAT assay were performed as for Fig. 2.

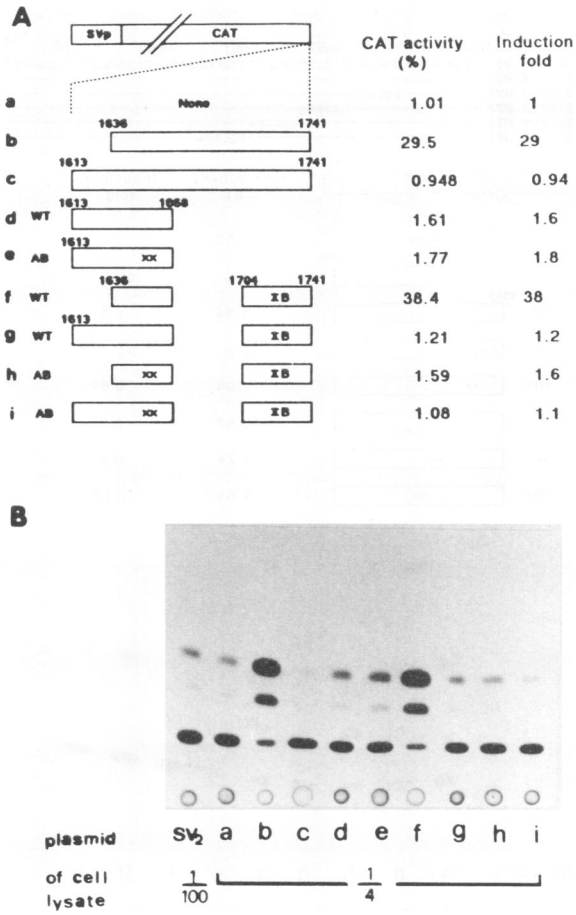

FIG. 5. Functional dependence of the NRE on an intact enhancer II. Different portions of the HBV sequences, either wild type (WT) or mutated, were cloned downstream of a CAT reporter gene driven by the SV40 early promoter. The transfection and CAT assays were performed as for Fig. 2. The different HBV sequences tested are as follows: a, none; b, enhancer II (nt 1636 to 1741); c, NRE plus enhancer II (nt 1613 to 1741); d, NRE plus box α (nt 1613 to 1668); e, NRE plus box α with AB mutation (nt 1613 to 1668 with AB mutation); f, box α plus enhancer IIB (nt 1636 to 1668 plus nt 1704 to 1741); g, NRE plus box α plus enhancer IIB (nt 1613 to 1668 plus nt 1704 to 1741); h, box α with AB mutation plus enhancer IIB (nt 1636 to 1668 with AB mutation plus nt 1704 to 1741); i, NRE plus box α with AB mutation enhancer IIB (nt 1613 to 1668 with AB mutation plus nt 1704 to 1741). (A) Plasmid constructs, normalized CAT activities, and fold induction. (B) Representative autoradiogram of the CAT assay.

support the notion that a direct repression of the promoter activity is not the major mechanism by which the NRE exerts its function.

Alternatively, the negative regulatory effect of the NRE results from a direct repression of enhancer II activity. It was shown previously that enhancer II is composed of two sequence motifs, box α and box β, and that cooperation of the two is required for the enhancer function (26). In line with what we have shown before (26), the sequence from nt 1636 to 1668 (box α) in conjunction with the sequence from nt 1704 to 1741 (enhancer IIB) which contains box β can activate the SV40 early promoter by 30-fold (box α plus IIB; Fig. 5B, lane f). The addition of the NRE, on the contrary, drastically represses the stimulation conferred by enhancer II (NRE plus box α plus IIB; lane g). Moreover, the repressive effect of the NRE is seen only when a functional enhancer II is present, in

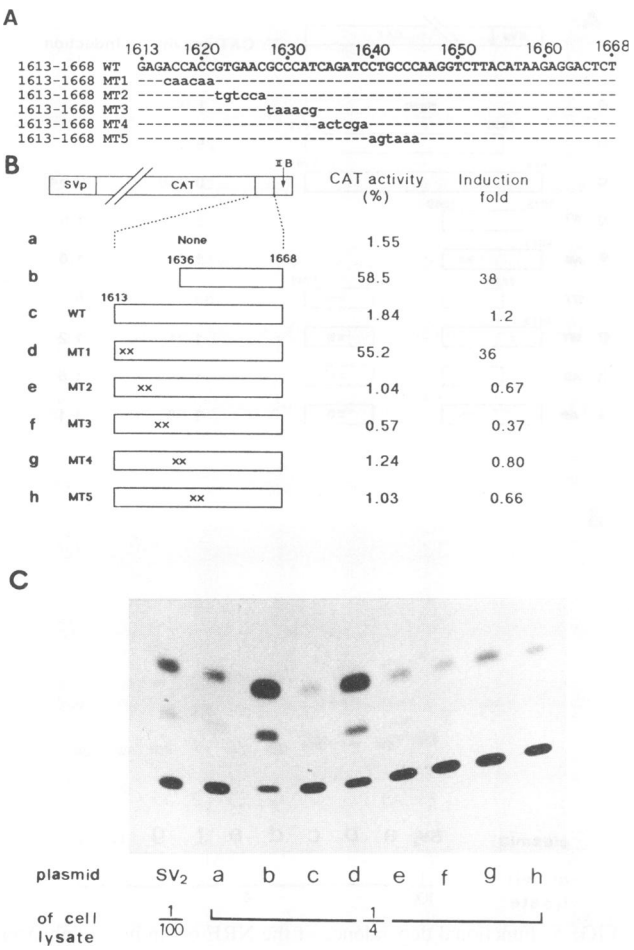

FIG. 6. Linker-scanning analysis of the NRE. The nucleotide sequence of the NRE was changed serially, and the effects of these changes on NRE function were analyzed by transient transfection and CAT assay. (A) Wild-type (WT) and mutant (MT1 through MT5) NRE sequences. (B) Plasmid constructs, normalized CAT activities, and the fold induction. These NRE plus box α sequences were linked with enhancer IIB and inserted downstream of a CAT reporter gene driven by an SV40 early promoter. The transfection and CAT assay were performed as for Fig. 2. (C) Representative autoradiogram of the CAT assay.

that neither NRE plus box α alone (nt 1613 to 1668; lane d) nor NRE + mutated enhancer II (NRE plus box α with AB mutation plus enhancer IIB; lane i) (25) is functional. These results demonstrate that the function of the NRE depends on an intact enhancer II and that the negative regulatory effect exhibited by the NRE comes from a direct repression of HBV enhancer II.

Identification of minimal essential elements for the NRE by mutant analysis. To precisely map the sequence required for the function of the NRE, we performed a linker-scanning analysis. Five different mutants were generated, and their repressive effects on enhancer II (box α plus enhancer IIB) were quantitated (Fig. 6). It turns out that nucleotide sequence substitutions over the region from nt 1622 to 1645 do not alter the function of the NRE whereas sequence changes over the segment from nt 1616 to 1621 completely destroy the repressive effect seen with the NRE. These results establish that the sequence from nt 1616 to 1621 is essential for the function of the NRE.

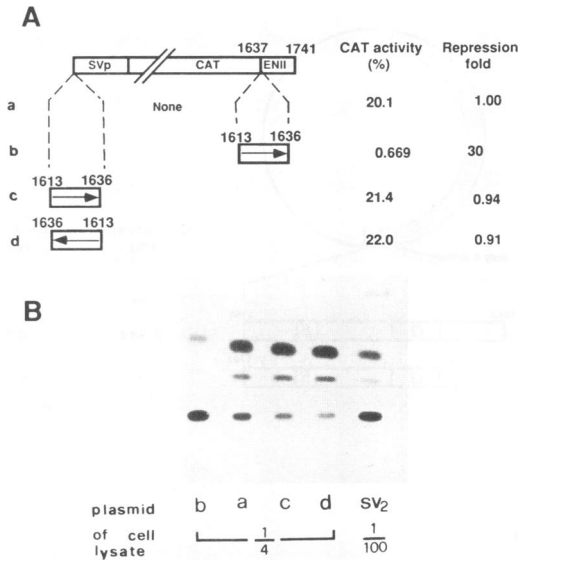

FIG. 7. Dependence of the repressive function of the NRE on its close proximity to enhancer II. The NRE sequence (nt 1613 to 1636) was placed in both orientations upstream of the SV40 early promoter of pA3SVpCAT(1636–1741), and its repressive effect on enhancer II was tested in transient transfections. (A) Plasmid constructs, normalized CAT activity, and fold repression. (B) Representative autoradiogram of the CAT assay. The DNA transfection and CAT assay were performed as for Fig. 2.

Distance dependence of NRE function on enhancer II. In the native organization of HBV, NRE is 27 bp away from enhancer II. It is interesting to investigate whether NRE can still repress enhancer II at a longer distance. Plasmids containing NRE upstream of the SV40 early promoter and enhancer II downstream of the CAT reporter gene were constructed. As shown in Fig. 7, NREs in both orientations do not repress the stimulating function of enhancer II when NRE is 1.8 kb away from enhancer II. Therefore, it is apparent that the repressive function of NRE on enhancer II is distance dependent.

Repression of enhancer II activity on the HBV surface and X promoters. We have previously shown that enhancer II can stimulate not only heterologous but also homologous promoters such as the surface and X promoters of HBV (25; our unpublished result). To understand whether the NRE has any functional role in the control of HBV gene expression, we then examined whether the NRE also modulates the viral promoters in the presence of the second enhancer. Both enhancer II and NRE plus enhancer II were positioned downstream of a CAT reporter construct driven by either the SPI or the X promoter of the HBV. As shown in Fig. 8, the NRE sequence leads to a complete loss of enhancer II activity on both surface and X promoters. These results indicate that the NRE is functional over endogenous viral promoters and may thus be involved in the control of HBV gene expression in vivo.

Gel shift analysis of NRE-binding proteins. A gel shift analysis was performed to examine the interaction of the cellular factor(s) with the NRE sequence. Crude nuclear extracts from HepG2 cells were loaded on a heparin-Sepharose column and step eluted with increasing concentrations of NaCl. Each fraction was then collected and tested for binding to the NRE sequence. When a double-stranded DNA (nt 1613 to 1636) containing NRE was used as a probe in the binding reaction, formation of two and one DNA-protein complexes was noted with the fraction eluted at 0.3 M and 0.4 M NaCl,

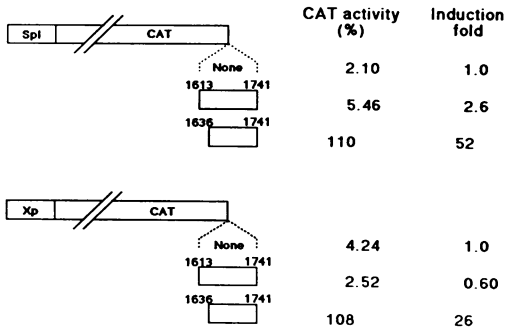

FIG. 8. Repression of the enhancer II stimulatory effects on HBV viral promoters by the NRE. HBV sequences containing enhancer II (nt 1636 to 1741) and NRE plus enhancer II (nt 1613 to 1741) were inserted downstream of a CAT reporter gene driven by either an HBV SPI or an HBV XP promoter, and their effects on transcription were measured by CAT assay. The transfection and CAT assay were performed as for Fig. 2. The various plasmid constructs, normalized CAT activities, and fold induction are shown schematically.

respectively. Other fractions did not show a clear DNA-protein complex band. The DNA-protein complexes at the 0.3 M NaCl fraction were nonspecific for NRE in that one could be inhibited by both 1613–1636WT and 1613–1668MT1 and the other could not be inhibited by either (data not shown). In contrast, the DNA-protein complex at the 0.4 M NaCl fraction appeared to be specific for NRE since it could be inhibited by 1613–1636WT, 1613–1668WT, and 1613–1668AB but not by 1613–1668MT1 (Fig. 9). Therefore, a specific NRE-binding protein(s) is present at the 0.4 M NaCl fraction.

DISCUSSION

We have previously reported the identification of a liver-specific enhancer, enhancer II, in the HBV genome (25).

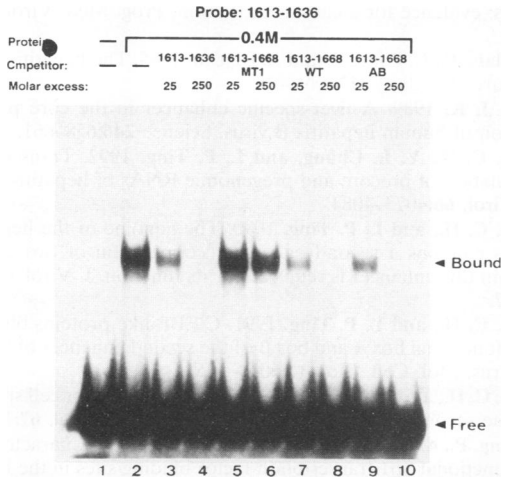

FIG. 9. Gel shift analysis of the functionally defined NRE sequence. The double-stranded DNA corresponding to nt 1613 to 1636 was labeled by Klenow fill-in and incubated with 10 μg of protein from the 0.4 M NaCl fraction of HepG2 nuclear extracts in the presence of no competitor (lane 2) or of increasing amounts of unlabeled 1613–1636WT sequence (lanes 3 and 4), 1613–1668MT1 (lanes 5 and 6), 1613–1668WT (lanes 7 and 8), and 1613–1668AB (lanes 9 and 10) at 30°C for 30 min. After incubation, the DNA-protein complexes were resolved on a native 4% polyacrylamide gel. The shifting band and probe are indicated as bound and free; lane 1 is a no-protein control.

Enhancer II is composed of two minimal essential sequence constituents, box α (nt 1646 to 1668) and box β (nt 1704 to 1715) (26). Interestingly, enhancer II coincides with the upstream regulatory sequence of the core promoter (24). In this study, we report the identification of an NRE (nt 1613 to 1635) that can repress the stimulatory effect of both the CURS and the second enhancer of HBV in differentiated liver cells. Moreover, the negative regulatory effect on enhancer II by the NRE is observed mainly in the context of a functional enhancer II. Linker-scanning analysis reveals that the sequence from nt 1616 to 1621 is required for NRE function, whereas modifications in the sequence from nt 1622 to 1645 do not seem to affect NRE function.

Analysis of HBV gene expression, like studies of eukaryotic genes, has generally focused on positive regulatory sequences and positive *trans*-acting factors. The discovery of the NRE is intriguing, since the addition of negative elements to positive regulatory regions may introduce extra levels of regulation. This may permit gene activity to be better adjusted in response to environmental demands, since transcription efficiency will now depend on the combined effects of not only positive regulatory elements but also NREs.

Several distinct modes of transcriptional repression have been proposed (12, 15, 18). In the competition mechanism, repressors may bind directly at or near the transcription start sites and compete for the formation of initiation complex in the promoter. This appears to account for the negative effect of SV40 large T antigen on its own promoter (19). Alternatively, activators and repressors may compete for overlapping or closely linked binding sites (14). The latter form of repression through competition has been invoked for the negative regulation of target genes by the members of the steroid hormone receptor superfamily (17). In the "quenching" mechanism, the repressor and activator bind to adjacent, nonoverlapping DNA sequences but the repressor neutralizes the ability of the activator to transmit stimulatory signals to the initiation complex. Such activator masking is seen in the regulation of expression of the neuroectoderm stripe genes in *Drosophila melanogaster*; in particular, the *sna* repressor can act over a distance of 7 to 25 bp to block activation by *dl* (10, 13). The fourth type of repression is mediated via silencers and can function in a position- and orientation-independent manner (15, 18).

The negative regulatory effect of NRE on HBV enhancer II is most likely to be mediated through activator masking for the following reasons: (i) neither the NRE (nt 1613 to 1636) nor the NRE plus box α (nt 1613 to 1668) alone has significant inhibitory effect on the SV40 early promoter; (ii) the function of the NRE is dependent on an intact enhancer II element; (iii) the essential element for the NRE as revealed in linker-scanning analysis is 25 nt away from box α; and (iv) the repressive function of the NRE on enhancer II is dependent on their close proximity. As for the effect of NRE on CURS, the situation is less clear. We have previously shown that multiple interacting elements are present within CURS (24). The repression on CURS by the NRE may thus be through the diminution of the stimulatory effects of one or many of these elements within CURS. In view of the dependence of the function of NRE on its close proximity to the enhancer II element (nt 1636 to 1741) when the latter functions as an enhancer, we suspect that NRE can repress the same sequence element, now functioning as CURS, with the same distance requirement.

Earlier study by Guo et al. did not observe the negative regulatory element that functions in differentiated liver cells as we defined in the current work (9). Since different deletion

mutants were used in these two studies, the discrepancy may be explained in the following ways: (i) the function of the NRE is sensitive to the sequence context (for example, see reference 11); and/or (ii) another positive regulatory element(s) may exist upstream that, directly or indirectly, counteracts the negative influence by the NRE. As can be seen in Fig. 2, the sequence from nt 1402 to 1851 exhibits much elevated transcriptional activity compared with that of nt 1613 to 1851. This and our earlier observation that the sequence between nt 1402 and 1804 exhibits strong enhancer activity (25) suggest that when sequence upstream of the NRE is included, either one or both of the above may come into play.

Incidentally, it has been shown that the transcription factor Sp1 binds to the sequence from nt 1623 to 1640 in HBV (28). The role of such interaction, however, remains to be elucidated. In our linker-scanning analysis, none of the mutants containing sequence changes from nt 1622 through 1645 displays transcriptional activity different from that of the wild type. This result indicates that in the context when enhancer II activity is repressed by the NRE, this particular Sp1 element does not appear to be indispensable.

In conclusion, we report the identification and characterization of the NRE that represses enhancer II function. It is increasingly apparent that HBV gene expression, like eukaryotic gene transcription, is subject to a highly complex interplay of both positive and negative interactions. Much of the current work on transcriptional regulation has concentrated on the operation of individual control elements. The ultimate goal should be to understand how multiple positive and negative control circuits function together to determine the level of viral gene expression during different phases of viral infection in vivo.

ACKNOWLEDGMENTS

We thank Shiuh-Wen Luoh for a critical review of and suggestions for improving the manuscript.

This research was supported by research grants NSC-82-0419-B010-090MB from the National Science Council and DOH82-HR-C08 from the National Institute of Health, Department of Health, the Executive Yuan, Republic of China.

REFERENCES

1. **Beasley, R. P., L. Y. Hwang, C. C. Lin, and C. S. Chien.** 1981. Hepatocellular carcinoma and hepatitis B virus: a prospective study of 22,707 men in Taiwan. Lancet **ii:**1129–1136.
2. **Bosch, V., C. Kuhn, and H. Schaller.** 1988. Hepatitis B virus replication, p. 43–58. *In* E. Domingo, J. J. Holland, and P. Ahlquist (ed.), RNA genetics, vol. 2. Retroviruses, viroids, and RNA recombination. CRC Press, Inc., Cleveland, Ohio.
3. **Chang, H. K., B. Y. Wang, C. H. Yuh, C. L. Wei, and L. P. Ting.** 1989. A liver-specific nuclear factor interacts with the promoter region of the large surface protein gene of human hepatitis B virus. Mol. Cell. Biol. **9:**5189–5197.
4. **De Medina, T., O. Faktor, and Y. Shaul.** 1988. The S promoter of hepatitis B virus is regulated by positive and negative elements. Mol. Cell. Biol. **8:**2449–2455.
5. **Ganem, D., and H. E. Varmus.** 1987. The molecular biology of the hepatitis B viruses. Annu. Rev. Biochem. **56:**651–693.
6. **Gorman, C., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
7. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
8. **Guo, W., K. D. Bell, and J. H. Ou.** 1991. Characterization of the hepatitis B virus EnhI enhancer and X promoter complex. J. Virol. **65:**6686–6692.
9. **Guo, W., M. Chen, T. S. B. Yen, and J. H. Ou.** 1993. Hepatocyte-specific expression of the hepatitis B virus core promoter depends on both positive and negative regulation. Mol. Cell. Biol. **13:**443–448.
10. **Ip, Y. T., R. E. Park, D. Kosman, E. Bier, and M. Levine.** 1992. The *dorsal* gradient morphogen regulates stripes of *rhomboid* expression in the presumptive neuroectoderm of the *Drosophila* embryo. Genes Dev. **6:**1728–1739.
11. **Jackson, D. A., K. E. Rowader, K. Stevens, C. Jiang, P. Milos, and K. S. Zaret.** 1993. Modulation of liver-specific transcription by interactions between hepatocyte nuclear factor 3 and nuclear factor 1 binding DNA in close apposition. Mol. Cell. Biol. **13:**2401–2410.
12. **Jackson, M. E.** 1991. Negative regulation of eukaryotic transcription. J. Cell Sci. **100:**1–7.
13. **Jiang, J., and M. Levine.** 1993. Binding affinities and cooperative interactions with bHLH activators delimit threshold responses to the *dorsal* gradient morphogen. Cell **72:**741–752.
14. **Keller, A. D., and T. Maniatis.** 1991. Identification and characterization of a novel repressor of β-interferon gene expression. Genes Dev. **5:**868–879.
15. **Levine, M., and J. L. Manley.** 1989. Transcriptional repression of eukaryotic promoters. Cell **59:**405–408.
16. **López-Cabrera, M., J. Letovsky, K. Q. Hu, and A. Siddiqui.** 1990. Multiple liver-specific factors bind to the hepatitis B virus core/pregenomic promoter: *trans*-activation and repression by CCAAT/enhancer binding protein. Proc. Natl. Acad. Sci. USA **87:**5069–5073.
17. **Mietus-Snyder, M., F. M. Sladek, G. S. Ginsburg, C. F. Guo, J. A. Ladias, J. E. Darnell, and S. K. Karathanasis.** 1992. Antagonism between apolipoprotein AI regulatory protein 1, Ear3/COUP-TF, and hepatocyte nuclear factor 4 modulates apolipoprotein CIII gene expression in liver and intestinal cells. Mol. Cell. Biol. **12:**1708–1718.
18. **Renkawitz, R.** 1990. Transcriptional repression in eukaryotes. Trends Genet. **6:**192–197.
19. **Rio, D., A. Robbins, R. Myers, and R. Tjian.** 1980. Regulation of simian virus 40 early transcription *in vitro* by a purified tumor antigen. Proc. Natl. Acad. Sci. USA **77:**5706–5710.
20. **Shaul, Y., W. J. Rutter, and O. Laub.** 1985. A human hepatitis B virus enhancer element. EMBO J. **4:**427–430.
21. **Szmumess, W.** 1978. Hepatocellular carcinoma and the hepatitis B virus: evidence for a causal association. Prog. Med. Virol. **24:**40–69.
22. **Tiollais, P., C. Pourcel, and A. Dejean.** 1985. The hepatitis B virus. Nature (London) **317:**489–495.
23. **Yee, J. K.** 1989. A liver-specific enhancer in the core promoter region of human hepatitis B virus. Science **246:**658–661.
24. **Yuh, C. H., Y. L. Chang, and L. P. Ting.** 1992. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. J. Virol. **66:**4073–4084.
25. **Yuh, C. H., and L. P. Ting.** 1990. The genome of the hepatitis B virus contains a second enhancer: cooperation of two elements within this enhancer is required for its function. J. Virol. **64:**4281–4287.
26. **Yuh, C. H., and L. P. Ting.** 1991. C/EBP-like proteins binding to the functional box α and box β of the second enhancer of hepatitis B virus. Mol. Cell. Biol. **11:**5044–5052.
27. **Yuh, C. H., and L. P. Ting.** 1993. Differentiated liver cell specificity of the second enhancer of hepatitis B virus. J. Virol. **67:**142–149.
28. **Zhang, P., A. K. Raney, and A. McLachlan.** 1993. Characterization of functional Sp1 transcription factor binding sites in the hepatitis B virus nucleocapsid promoter. J. Virol. **67:**1472–1481.
29. **Zhou, D. X., and T. S. B. Yen.** 1991. The ubiquitous transcription factor Oct-1 and the liver-specific factor HNF-1 are both required to activate transcription of a hepatitis B virus promoter. Mol. Cell. Biol. **11:**1353–1359.
30. **Zhou, D. X., and T. S. B. Yen.** 1991. The hepatitis B virus S promoter comprises a CCAAT motif and two initiation regions. J. Biol. Chem. **266:**23416–23421.