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# Characterization of a Positive Regulatory *cis*-Element and Transacting Factors for the Hepatitis B Viral Pregenomic Promoter

**Cheol Yong Choi, Geon Tae Park and Hyune Mo Rho***

*Department of Molecular Biology and Research Center for Cell Differentiation, Seoul National University, Seoul 151-742, Korea*

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**Abstract:** Transcription of hepatitis B viral pregenomic promoter is known to be regulated mainly by the combined interaction of enhancers I, II and the intervening regulatory sequences between the two enhancers. A positive regulatory element was identified by serial deletion and measuring the linked chloramphenicol acetyltransferase (CAT) activities, which overlapped with the 5′ region of the X open reading frame. When the positive regulatory element was inserted upstream of the SV40 early promoter, it elevated SV40 promoter activity in HepG2 cells. Two cellular proteins of 110 (p110) and 33 (p33) kDa interacted with the positive element and both of them were present in the nucleus, but p110 also existed in the cytoplasm in phosphorylated form. Dephosphorylation of p110 by acid phosphatase enhanced the DNA-binding activity of p110. The p33 could bind to single-strand DNA specifically as well as to double-strand DNA.

**Key words:** hepatitis B virus, positive element, pregenomic promoter.

Human hepatitis B virus (HBV) causes chronic and acute infection of the liver and the infection has been linked with hepatocellular carcinoma. It is the unique character of HBV, unlike other DNA viruses, that it encodes reverse transcriptase (Ganem and Varmus, 1987; Shin and Rho, 1995) and replicates through reverse transcription of a pregenomic RNA (Summers and Mason, 1982). Therefore, the transcriptional regulation of pregenomic promoter may play a key role in the replication and viral life cycle. Transcription of HBV pregenomic promoter is known to be regulated mainly by the combined interaction of enhancer I (EnI), enhancer II (EnII) and the intervening regulatory sequence between the two enhancers. EnI is located within the viral polymerase gene and is upstream of the X gene promoter. The functional organization of EnI has been investigated in some detail (Dikstein *et al.*, 1990; Faktor *et al.*, 1990). It has previously been shown that EnI positively regulated the level of transcription from pregenomic promoter approximately two- to ten-fold (Zhang *et al.*, 1992). EnII is located within the X open reading frame, about 400 bp downstream of EnI and is highly liver specific, functioning only in highly differentiated human hepatoma cells (Yaginuma and Koike, 1989; Wang *et al.*, 1990). Furthermore, EnII activity varies in different hepatoma lines, suggesting that this enhancer is regulated according to the differentiated state of the hepatoma line used (Yee, 1989; Yuh and Ting, 1990).

In the HBV genome, the space region between EnI and EnII occupies about 400 base pair DNA sequence. The sequence between EnI and EnII activates transcription from pregenomic promoter approximately three- to four-fold in Huh7, Hep3B, and HepG2.1 cells, but the sequence mediating this effect was not precisely identified (Zhang *et al.*, 1992). This result suggests that additional regulatory elements may be present between the EnI and EnII/pregenomic promoter. It was shown by DNase I footprinting analysis that some cellular proteins bind to the region (Guo *et al.*, 1993). In addition, HNF4 (Guo *et al.*, 1993) and Sp1 (Zhang *et al.*, 1993) binding sites and a negative regulatory element (Lo and Ting, 1994) were also reported in the immediate upstream of EnII. In this regard, it has been believed that the intervening sequence between EnI and EnII /pregenomic promoter plays some functional role in the regulation of pregenomic promoter.

In this report, we present data that demonstrate the presence of a positive regulatory element which has 67% homology with the retinoblastoma control element (RCE) of the *c-fos* gene promoter. Cellular proteins of 110 kDa and 33 kDa binding to the positive element were identified. The functional role of this element is

*To whom correspondence should be addressed.
Tel: 82-2-880-6688, Fax: 82-2-872-1993.

also discussed.

## Materials and Methods

### Plasmids construction

All plasmids were constructed by standard DNA cloning procedures (Sambrook *et al.*, 1989). Plasmids with a 5′ deletion of the upstream region of the pregenomic promoter region were obtained from pBACAT containing the *Bam*HI-*Alu*I DNA fragment (nt 1397 to 1873) of the HBV genome (Rho *et al.*, 1989) in front of the CAT gene by digestion with *Sac*II (pSACAT), *Xma*III (pXACAT), respectively. For the construction of pCATHX, pCATBS, pCATSX, the *Bam*HI-*Xma*III DNA fragment (nt 1397 to 1507) of the HBV genome was eluted and digested with *Hae*II or *Sac*II, and the resulting fragments were inserted upstream of the simian virus (SV) 40 early promoter, at the *Bgl*II site of pCAT-promoter (Promega Corporation, Madison, USA). The plasmids pBLcat2XPRE and pBLcat4XPRE were obtained by introducing the multimerized (two and four copies, respectively) synthetic oligonucleotide corresponding to the positive element into the *Bam*HI site of pBLcat2 (Luckow and Schütz, 1987). The plasmids pCATpro5′PRE and pCATpro3′PRE were obtained by introducing the two copies of the positive element oligonucleotide into the *Bgl*II and *Bam*HI sites of pCATpromoter (Promega Corporation, Madison, USA), respectively.

### Cell culture and transfection

Penicillin (100 U/ml) and streptomycin (100 μg/ml) were added to all media. All cell lines were maintained in an environment of 5% $CO_2$ at 37℃. HepG2 cells (differentiated human liver cell), SK-Hep1 (undifferentiated human liver cell), and lung carcinoma cells (A 549) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cell cultures were transfected with plasmid DNA using the calcium phosphate precipitate formed in BES (N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid) buffer (Kim *et al.*, 1992a). Approximately, $1.5\times10^6$ cells were plated 24 h prior to transfection. Before addition of the DNA mixture, the medium was replaced with 5 ml of fresh medium containing serum. The solution of calcium chloride, plasmid DNA including pRSV-βgal reporter and each effector plasmid (total 20 μg), and BES buffer (pH 6.95) were added to a plate. The plate was incubated for 18 h at 37℃, 3% $CO_2$, the cells were washed with phosphate buffered saline (PBS) for removing the DNA and buffer, and then incubated at 37℃ for an additional 30 h in the fresh growth medium. The cells were harvested for enzymatic assay after a total of 48 h incubation.

### CAT assay

CAT assay was performed as described previously (Gorman *et al.*, 1982). The transfected cells were harvested and lysed by three cycles of freezing and thawing. One fourth of the cell lysate was used to determine β-galactosidase activity. Extracts were incubated with 0.2 μCi of [$^{14}$C]-labeled chloramphenicol and 20 μl of 4 mM acetyl coenzyme A in 0.5 M Tris-Cl (pH 7.6) at 37℃ for 2 h. After incubation, the reaction mixture was extracted with ethylacetate, vacuum dried for concentration, and then loaded on silica gel chromatography plates and chromatographed in a chloroform and methanol solution (95:5). The plates were air dried and subjected to autoradiography and the amount of acetylated [$^{14}$C]-chloramphenicol was measured by scintillation counting.

### Preparation of nuclear extracts and mobility shift assay

Nuclear extracts from HepG2, SK-Hep1, A549, and HeLa cells were made as described (Dignam *et al.*, 1983; Andrews and Faller, 1991). The probe for mobility shift assay was synthesized. The sequence is 5′-GATCCCGCGGACGACCCGTCTCGCGGCCGTTTGA-3′. The probe was end-labeled with [γ-$^{32}$P]ATP and kinase. Nuclear extracts were mixed with a radioactive probe in the presence or absence of a 100-fold molar excess of cold probe for 20 min at 20℃ in a 15 μl solution containing 10 mM Hepes, 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 2 μg of poly(dI-dC) (Sigma Chemical Co.). Samples were electrophoresed in 6% acrylamide gels in 0.5 X TBE (44 mM Tris, 44 mM boric acid, and 1 mM EDTA). After electrophoresis, gels were dried and exposed to X-ray film. For the elution of proteins, nuclear extracts were electrophoresed. Each of the p110 and p33 bands was excised from the gel and chopped into small pieces. Proteins were eluted electrophoretically in a buffer containing 150 mM NaCl, 20 mM Hepes (pH 7.5), 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mg of BSA per ml. The eluted protein was renatured by dialysis against 100 ml of elution buffer containing 20% glycerol for 2 h at 4℃. The protein samples were then assayed for PRE-binding activity by a mobility shift assay.

### UV-crosslinking analysis

The 5′-end-labeled PRE probe was incubated with HepG2 nuclear extract under standard mobility shift assay conditions in the presence of 500 ng of poly (dI-dC) in a 10 μl binding reaction at room tempera-

ture. The reaction mixture was transferred onto a microplate, and UV irradiated with a transilluminator (254 nm, 2400 microwatts/cm$^2$) for 10 min at 4℃. 100 mM DTT (final concentration) was added prior to electrophoresis on a 10% SDS-polyacrylamide gel. The gel was fixed and dried under a vacuum, and the proteins directly involved in the DNA interaction were identified by autoradiography. For the dephosphorylation of proteins, nuclear and cytoplasmic extracts were incubated with 40 milliunits of potato acid phosphatase (Sigma Chemical Co., St. Louis, USA) for 20 min at 25℃.

## Results

### The upstream region of the pregenomic promoter of HBV contains a positive regulatory element

We examined whether a sequence upstream of the pregenomic promoter carries elements modulating the promoter activity. To investigate the effect of the region between enhancer I and enhancer II on the pregenomic promoter activity, a series of 5′ deletions from the *Bam*HI site (−416, relative to the transcription start site, Will *et al.*, 1987; Yaginuma *et al.*, 1987) to the *Xma*III site (−306) was constructed and used to direct the expression of chloramphenicol acetyltransferase (CAT) reporter gene. These plasmids were transfected into HepG2 cells and the level of CAT activity was determined 48 h posttransfection (Fig. 1). The initial plasmid contains the HBV sequence from the *Bam*HI site (−416) to the *Alu*I site (+62) in front of the CAT gene. Deletion of sequences to the *Sac*II site (−368) resulted in a slight increase in CAT activity. Further deletion of DNA sequences to the *Xma*III site (−306) resulted in about a three-fold decrease in CAT activity in HepG2 cells (Fig. 1, pXACAT). These results indicated that there is a positive regulatory element in the *Sac*II-*Xma*III (nt 1446~1464) DNA fragment.

To substantiate the positive regulatory element, the effect of this element on the heterologous promoter was examined. The *Bam*HI-*Xma*III (nt 1397~1507) DNA fragment was digested with *Hae*II or *Sac*II and the resulting fragments were inserted upstream of the simian virus 40 early promoter (Fig. 2) and these plasmids were transfected into HepG2 cells and the level of CAT activity was determined as previously (Fig. 2). The plasmids containing the *Hae*II-*Xma*III and *Sac*II-*Xma*III fragment showed about eight- and ten-fold increase in CAT activity, respectively, in HepG2 cells (Fig. 2, pCATHX and pCATSX). Therefore, *Sac*II-*Xma*III DNA fragment may contain a positive regulatory element. These results exactly coincide with the deletion analysis performed previously.

### Two cellular proteins interact with the positive regulatory element

The putative positive regulatory sequence is well conserved among the HBV subtypes (Fujiyama *et al.*, 1983; Valenzuela *et al.*, 1980; Galibert *et al.*, 1979) and has comparable homology with the Rb control element (RCE) of the *c-fos* gene promoter (Robbins *et al.*, 1990). Based on the sequence matched with *c-fos* RCE, we synthesized an oligonucleotide corresponding to the positive regulatory element (PRE) and subsequently performed a mobility shift assay with HepG2 nuclear extract. Nuclear extracts from HepG2 cells were incubated with $^{32}$P-labeled oligonucleotides and subjected to 6% polyacrylamide non-denaturing electrophoresis. Two specific DNA-protein complexes were formed with a positive element probe (Fig. 3). These results show that two kinds of nuclear proteins interact with

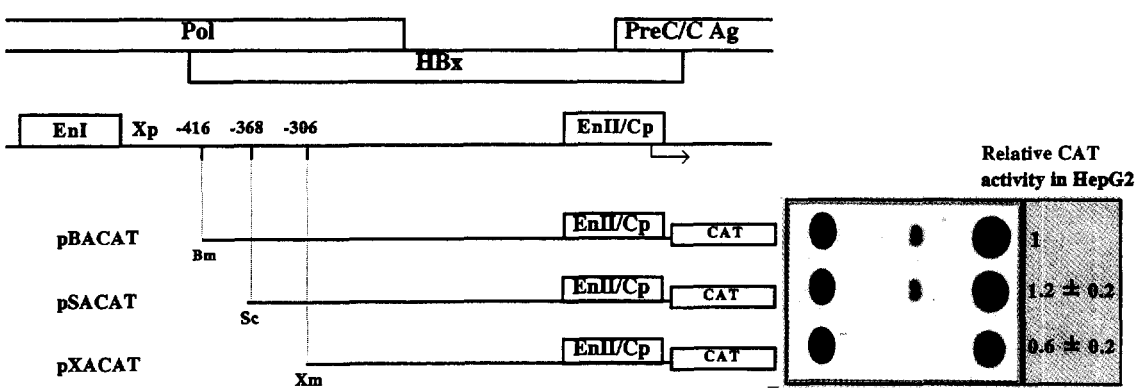


**Fig. 1.** Serial deletion analysis of the upstream region of pregenomic promoter. Initial plasmid (pBACAT) contains *Bam*HI-*Alu*I (−416 to +62) DNA fragment in front of the CAT reporter gene. The DNA fragment from the *Bam*HI site to *Sac*II, *Xma*III, sites were deleted and the resulting plasmids designated as pSACAT, pXACAT, respectively. Schematic map and ORFs of HBV are shown on the top of the figure. Each of these plasmids was transfected into HepG2 cells that were assayed for CAT activity as described in Materials and Methods. Relative CAT activities in HepG2 cells are shown on the right margin of figure. Abbreviations: Bm, *Bam*HI; Sc, *Sac*II; Xm, *Xma*III.

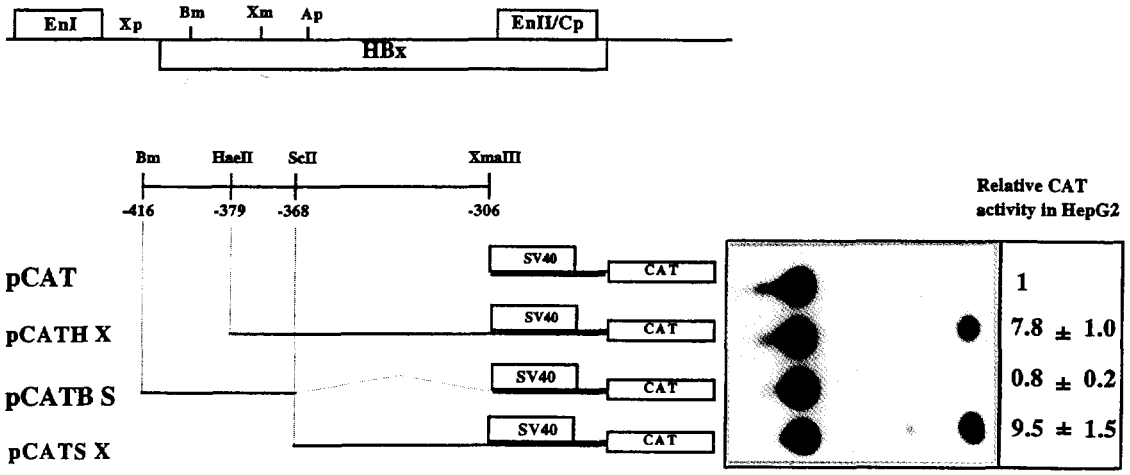


**Fig. 2.** Effect of the *Bam*HI-*Xma*III DNA fragments on the heterologous promoter, SV40 early promoter. The 106-bp *Bam*HI-*Xma*III fragment was excised from HBV genome and digested with *Hae*II or *Sac*II, and the resulting DNA fragments were inserted upstream of SV40 promoter. Initial plasmid is pCATpro (promega). The *Hae*II-*Xma*III, *Bam*HI-*Sac*II and *Sac*II-*Xma*III DNA fragments are positioned into the *Bgl*II site of pCATpro and designated as pCATHX, pCATBS, pCATSX, respectively. Schematic map of HBV genome is shown on the top of the figure. The restriction sites which have been used during the cloning procedures are indicated. One typical CAT assay in HepG2 cells transfected with the vectors is indicated. Relative CAT activities in HepG2 cells are shown on the right margin of figure. Abbreviations: Bm, *Bam*HI; HII, *Hae*II; ScII, *Sac*II; Xm, *Xma*III.

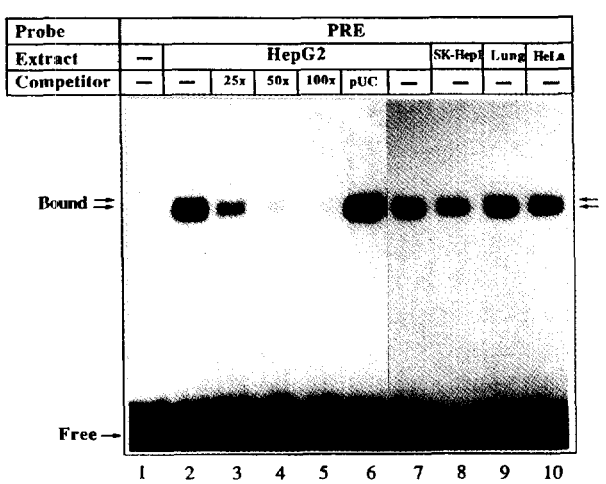


**Fig. 3.** Mobility shift assay with oligonucleotide corresponding to the positive regulatory element. The probe corresponding to the positive element was labeled at its 5'-end and incubated with HepG2 nuclear extracts prior to 6% polyacrylamide gel electrophoresis in 0.5 X TBE gel. Mobility shift assay was carried out in the presence of increasing amounts of unlabeled probe, 25X, 50X, 100X, respectively. Nuclear extracts derived from HepG2, SK-Hep1, A549 lung cell and HeLa cells were incubated with a radiolabeled probe.

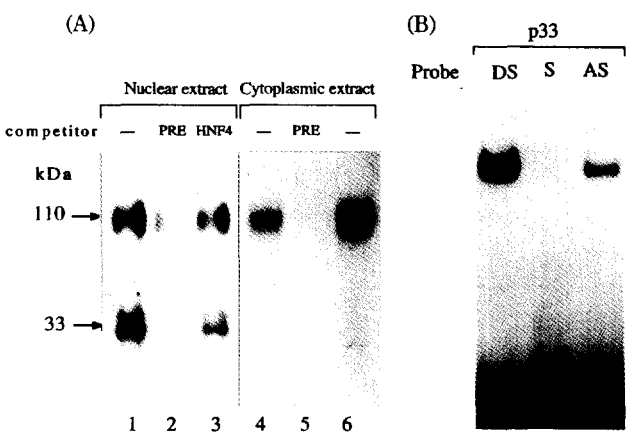


**Fig. 4.** Characterization of PRE-binding proteins. A: UV-crosslinking assay was carried out with HepG2 nuclear extracts (lanes 1, 2 and 3) and cytoplasmic extracts non-treated (lanes 4 and 5) or treated with potato acid phosphatase (lane 6). The standard binding reaction was performed using 5'-end-labeled PRE probe in the absence or presence of competitor as indicated in figure, and the DNA-protein complexes were crosslinked by UV irradiation and analyzed by 10% SDS-polyacrylamide gel electrophoresis. B: p33 was eluted from acrylamide gel and renatured as described in Materials and Methods, and then was used in a mobility shift assay with double strand (DS), upper (S) and lower strand (AS) of PRE oligonucleotide, respectively, as a probe.

the positive element. The complexes were abolished in the presence of a 100-fold molar excess of an unlabeled probe (Fig. 3, lane 5) but not with nonspecific DNA fragments, pUC19 digested with HaeIII. (Fig. 3, lane 6). To know whether the proteins binding to the positive element exhibit cell-line specificity, a mobility shift assay was performed with nuclear extracts derived from HepG2, differentiated human liver cell, SK-Hep1, undifferentiated human liver cell, A549, lung carcinoma cell, and HeLa, human cervical carcinoma cell. The positive element oligonucleotide formed identical DNA-protein complexes with all nuclear extracts tested (Fig. 3, lanes 7~10). These results suggested that the proteins binding to the positive element may be ubiquitous in liver or non-liver cells.

To characterize and determine the molecular size of these proteins, UV-crosslinking analysis was carried out with HepG2 nuclear extract (Fig. 4). The 5'-end-labeled PRE probe was incubated with HepG2 nuclear extract under standard mobility shift assay conditions, and UV was irradiated prior to electrophoresis on a 10% SDS-polyacrylamide gel. Two distinct DNA-protein complexes (132 and 55 kDa) were detected with HepG2 nuclear extract (Fig. 4, lane 1). In consideration of the molecular weight of the probe (double strand 34-mer, 22 kDa), the molecular weight of the DNA-binding proteins was estimated to be 110 and 33 kDa, respectively. The bands were abolished in the presence of a 100-fold molar excess of unlabeled PRE oligonucleotides (lane 2), but not with HNF4 oligonucleotides (lane 3). These results indicated that two cellular proteins of 110 and 33 kDa interact with the positive element.

### Characterization of the PRE-binding proteins

Given that the ratio of 110 kDa (p110) to 33 kDa (p33) in the course of nuclear extract preparation has been variable, the subcellular distribution of p110 and p33 was studied. When cytoplasmic extracts were used in the UV-crosslinking assay, p110 was found to be in the cytoplasmic fraction as well as in the nuclear extract at a comparable level (Fig. 4A, lane 4), and the band was competed out with cold probes (lane 5). Since many cytoplasmic transcription factors are regulated by phosphorylation (Hunter and Karin, 1992), it was also tested whether phosphorylation/dephosphorylation is involved in the function of p110. A UV-crosslinking assay was carried out with cytoplasmic extract treated with potato acid phosphatase. Dephosphorylation of p110 in the cytoplasmic extract enhanced the DNA-binding property of p110 (lane 6). These results suggested that p110 in the cytoplasm is a phosphorylated form which has lower DNA binding affinity. To further characterize the p110 and p33, a single-strand DNA binding property was tested. Following denaturation and renaturation of eluted p110 and p33, each protein was used in the mobility shift assay using the single-strand PRE oligonucleotide as a probe. Both double strand and antisense strand of PRE bound to p33. In contrast, no binding was detected using the sense strand (Fig. 4B). Also, any single-strand oligonucleotide was not bound to p110 (data not shown). A similar result has been reported for YB1 (Grant and Deeley, 1993) and estrogen receptor (Lannigan and Notides, 1989). However, the functional implication of the single-strand DNA binding property of p33 is not clear.

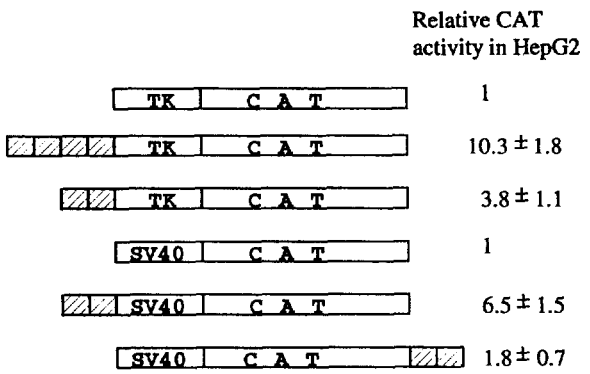


**Fig. 5.** Effect of positive regulatory element on the heterologous promoter, simian virus 40 early (SV40) and herpes simplex virus thymidine kinase (tk) promoter. HepG2 cells were transfected with plasmids that were under the control of the tk promoter that contained two or four copies of the positive element and under the control of the SV40 early promoter that contained two copies of positive element in 5' and 3' position. Each of these plasmids was transfected into HepG2 cells that were assayed for CAT activity as previously. Relative CAT activities are shown on the right margin of figure.

### The positive regulatory element transactivates the SV40 early promoter and thymidine kinase promoter activity

To investigate the transactivation activity of the positive element, synthetic oligonucleotides corresponding to the element were multimerized and progressive copy numbers were cloned upstream of the TK promoter (Fig. 5). The activities of these plasmids were assayed in HepG2 cells. In agreement with our above observation, the positive element had strong transactivation activity in HepG2 cells. This activity correlated linearly with the number of copies of the positive element. To assess the position effect of the positive element and the effect on the SV40 promoter , two copies of PRE were placed at 5' and 3' positions to the SV40 early promoter and the activities of these plasmids were assayed as described (Fig. 5). The positive element transactivated the SV40 promoter activity in the 5' position but to a lesser extent in the 3' position. Therefore, the positive regulatory element can transactivate the activity of heterologous promoters such as TK and SV 40 early promoter in a position dependent manner.

## Discussion

Transcription of HBV pregenomic promoter is known to be regulated mainly by the combined interaction of enhancer I (EnI), enhancer II (EnII) and the intervening regulatory sequences between EnI and EnII. Also, the activity of pregenomic promoter was elevated by

X or elongated X protein through the C/EBP binding sites (Choi *et al.*, 1993; Shim *et al.*, 1993). However, it is clear that the relative contribution of these regulatory regions to the transcriptional activity from the pregenomic promoter is cell type dependent (Zhang *et al.*, 1992). The functional organization of EnI and EnII has been investigated in some detail (Dikstein *et al.*, 1990; Faktor *et al.*, 1990; Wang *et al.*, 1990; Yaginuma *et al.*, 1989; Yee, 1989; Yuh and Ting, 1990). Some positive and negative regulatory elements influencing the activity of pregenomic promoter within the intervening regulatory region between EnI and EnII were reported. A negative regulatory element in the immediate upstream of EnII which can repress EnII function was identified and characterized (Lo and Ting, 1994). But HNF4 (Guo *et al.*, 1993) and Sp1 (Zhang *et al.*, 1993) were also known to be associated with this region and they transactivate pregenomic promoter activity. The meaning of the existence of these positive and negative regulatory elements with close proximity to the expression of pregenomic promoter remains to be understood. The HBV positive regulatory element of the pregenomic promoter, identified in this study, was located far upstream of EnII. This positive element may exert some function for the fine tuning of transcriptional regulation of the pregenomic promoter, or provide response to multiple stimuli. The other possibility is that the positive element may have an effect on the HBx promoter, located upstream of this element, and/or on the intragenic promoter in the X-ORF. Recently, novel short transcripts of HBV X gene from intragenic promoter were reported (Zheng *et al.*, 1994). Transcription start sites of the short transcripts were multiple and spanned from nt 1527 to nt 1629. The positive element is located upstream of the intragenic promoter, −53 relative to the start site of the longest small X transcript. It is speculated that the positive element identified in this study may participate positively in intragenic promoter activity. Functional analysis of the positive element on the intragenic promoter is ongoing. The sequence of the positive regulatory element has an overall 67% homology with the retinoblastoma control element (RCE) of the *c-fos* gene over 30-bp DNA sequences. The *c-fos* RCE was identified as being necessary for Rb-mediated transcription repression in NIH3T3 cells (Robbins *et al.*, 1990). Thereafter, RCE was found in the *c-myc* (Pietenpol *et al.*, 1991), transforming growth factor β1 (Kim *et al.*, 1991) and Insulin-like growth factor II promoter (Kim *et al.*, 1992b). Among the DNA binding proteins of *c-fos* RCE, Sp1 and Sp1 related proteins were characterized. The positive element is likely to be similar to *c-fos* RCE on the basis of the data that DNA-binding proteins of the positive element also had a binding property to *c-fos* RCE and purified Sp1 could bind to the positive element as well as *c-fos* RCE (data not shown). For the functional analysis of p110 and p33, a mutation study was performed. Mutation of the binding site of p110 or p33 showed that p33 participates directly in the transactivation, whereas p110 has an inhibitory effect on the function of p33. We postulate that alleviation of the p110-mediated inhibitory effect would lead to full activation of the positive element. These data will be published elsewhere. Further characterization and cDNA cloning of p110 and p33 are now being performed.

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