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Characterization of a Specific Region in the Hepatitis B Virus Enhancer I for

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Hepatitis B virus (HBV) enhancer I has been shown to consist of several *cis*-acting sequences for the HBV gene expression efficiently in certain types of cells. Transcriptional regulation of HBV X gene mediated by enhancer I might be one of the mechanisms by which HBV obtains hepatotropism. By mutagenesis analysis of enhancer I function in the enhancer I/X gene promoter complex, we characterized a specific transcriptional regulatory region (designated as a LSR element, nt 989–1030) of enhancer I for the X gene promoter by means of the transient transfection technique using hepatic and nonhepatic cells. Based on the analysis of protein factors interacting with the LSR element, liver-enriched transcriptional factors, HNF3 and HNF4 or retinoid X receptor α (RXRα), are probably implicated in the activity of enhancer I for the efficient expression of X gene through their interaction with the LSR element in the hepatic cell. Furthermore, the isolated LSR element was demonstrated to function alone as a specific *cis*-acting element and to be able to activate transcription from the X gene promoter efficiently in the hepatic cell in an orientation-independent manner.

INTRODUCTION

Hepatitis B virus (HBV) infection, mainly restricted to hepatocytes, subsequently causes acute and chronic hepatitis, and is thought to be closely related to the development of hepatocellular carcinoma (Beasley *et al.,* 1981; Tiollais *et al.,* 1981). HBV is a small DNA virus with a partially double-stranded 3.2-kb genome. The viral genome consists of four open reading frames coding for surface, core, polymerase, and X proteins (Ganem and Varmus, 1987). A series of genetic analyses has also revealed that the genome contains four promoters and two enhancers as *cis*-acting elements (Shaul *et al.,* 1985; Tognoni *et al.,* 1985; Yee, 1989; Schaller and Fischer, 1991). Enhancer I is located upstream of X gene and the X promoter region (Shaul *et al.,* 1985; Tognoni *et al.,* 1985; Jameel and Siddiqui, 1986). We have previously defined X promoter binding protein(s) (termed X-PBP), which binds to the immediate downstream region (nt 1097–1119) of enhancer I and the minimal X promoter sequence (nt 1102–1122) lacking homology to TATA box or GC box (Nakamura and Koike, 1992; Yaginuma *et al.,* 1993; Takada *et al.,* 1996). We also demonstrated that the element containing X-PBP binding site regulates the activity of X promoter (Nakamura and Koike, 1992; Yaginuma *et al.,* 1993). Enhancer II has been more recently demonstrated to be located upstream of and overlaps the core promoter region (Yee, 1989; Yuh and Ting, 1990). While both enhancer I and II have been shown to exhibit preference for hepatic cells (Shaul *et al.,* 1985; Jameel and Siddiqui, 1986; Antonucci and Rutter, 1989; Yee, 1989; Su and Yee, 1992; Yuh *et al.,* 1992; Yuh and Ting, 1993), the transcriptional activities of the core and X gene promoters have been demonstrated to be mostly regulated by enhancer I (Jameel and Siddiqui, 1986; Roossinck *et al.,* 1986; Antonucci and Rutter, 1989; Lopez-Cabrera *et al.,* 1990). Nevertheless, the determinant of the liver-specific transcriptional activity of enhancer I has not yet been fully analyzed in the context of the enhancer I/X promoter complex. Previous studies have shown that multiple *trans*-acting cellular factors bind to enhancer I sequences and some of those proteins are liver-enriched, however, some are ubiquitous factors (Fig. 1A) (Ben-Levy *et al.,* 1989; Ostapchuk *et al.,* 1989; Patel *et al.,* 1989; Dikstein *et al.,* 1990; Faktor *et al.,* 1990; Lopez-Cabrera *et al.,* 1990; Trujillo *et al.,* 1991; Huan and Siddiqui, 1992; Garcia *et al.,* 1993; Reith *et al.,* 1994; Ori and Shaul, 1995). The expression of a number of liver-specific genes has been demonstrated to be regulated by interactions between some liver-enriched transcriptional factors, such as HNF1, HNF3, HNF4, and C/EBP (Mietus-Snyder *et al.,* 1992; Gregori *et al.,* 1993; Metzger *et al.,* 1993; Fuernkranz *et al.,* 1994; Nishiyori *et al.,* 1994; Yanuka-Kashles *et al.,* 1994). On the basis of these findings, some of these liver-enriched protein factors are expected to be involved in the activity of enhancer I for the X gene expression in the hepatic cell.

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In this study, we characterized a specific region (designated as the LSR element, nt 989–1030) of enhancer I for the efficient transcription of X gene based on the deletion analysis in the context of the enhancer I/X promoter system transiently transfected into hepatic or nonhepatic cells. The LSR element contains two protein binding sites (2C/TGT3b and GB/RARE), to which HNF3 (Chen *et al.,* 1994; Ori and Shaul, 1995) and members of steroid hormon receptor superfamily, such as HNF4, RXRα, PPAR, and COUP-TFs bind *in vitro* (Siddiqui, 1995), respectively. Point mutations introduced in these sites resulted in substantial diminution of the transcriptional activity of enhancer I in the hepatic cell, but not in nonhepatic cells, indicating that these transcription factors might be implicated in the efficient transcription of X gene in the hepatic cell via their interaction with the LSR element of enhancer I. Furthermore, the isolated LSR element was also demonstrated to function alone as a *cis*-acting element, which is able to activate transcription from the X gene promoter efficiently in the hepatic cell.

MATERIALS AND METHODS

Plasmid DNAs

Deletion mutations in the HBV enhancer I region [nucleotides (nt) 989–1096, HBV subtype adr (Kobayashi and Koike, 1984)] were generated from the parental plasmid, pSPT18X, which contains both enhancer I and X promoter region (nt 989–1250). The parental plasmid was linealized within the enhancer I region and subsequently deleted by the standard procedure using exonuclease III and Mung bean nuclease (Takara Shuzo) and then recirculurized. Fragments containing X promoter region and enhancer I with various deletions in size were cut out from recirculurized plasmids and both ends of each fragment were ligated to *Hin*dIII linkers in order to introduce it into the *Hin*dIII site of the expression vector, pSV00CAT (Nippon Gene), which contains neither transcriptional promoter nor enhancer element except chloramphenicol acetyltransferase (CAT) gene.

Point mutations in the LSR element of enhancer I sequences of pXEPCAT were generated by using the PCR technique. Each construct was confirmed by DNA sequencing. The LSR-WT, LSR-2Cm, LSR-GBm, LSR-2CmGBm oligonucleotides are shown in Fig. 3A. These fragments were then cloned into 53 bp upstream the ApaLI site (nt 4591 of pSV00CAT) of the expression vector, pXM4CAT, that contains only HBV X promoter element, M4 (nt 1102–1141) (Takada *et al.,* 1996), upstream of the CAT gene.

Cells and transfection

HuH7 and HepG2 cells derived from human hepatocellular carcinoma and human hepatoblastoma, respectively, were cultured in DM-160AU (Kyokuto) containing 10% fetal bovine serum. Saos2 cells derived from human osteosarcoma were grown in DMEM (Nissui) containing 10% fetal bovine serum. NIH3T3 cells were grown in DMEM containing 10% calf serum.

Transient transfections of plasmid DNAs were performed by the calcium phosphate precipitation procedure (Graham and Van-Der-Eb, 1973). Cells were split and plated the day before transfection at the appropriate concentration for each cell line. The following day, cells were cotransfected with a construct of CAT reporter plasmid DNA and β-galactosidase control plasmid, pCMV-β (Clontech). The cells were incubated for 6 hr with calcium phosphate precipitate and then treated with 10% glycerol containing medium for 2.5 min. Fresh medium was then added and total cell extracts were prepared 48 hr later. CAT assays and β-gal assays were performed according to the published protocols (Gorman *et al.,* 1982; Herbomel *et al.,* 1984).

Nuclear extract preparation and electrophoretic mobility shift assay

Nuclear extracts were prepared according to the method of Dignam *et al.* (1983). Electrophoretic mobility shift assays (EMSA) were performed in a final volume of 15 μl containing 5 μg of nuclear extract, 10 m*M* Tris–HCl (pH 7.5), 50 m*M* NaCl, 1 m*M* dithiothreitol, 1 m*M* EDTA, 5% glycerol, 200 ng of poly (dI-dC) per μl, and 20,000 cpm of probe (10–30 fmol). The binding reactions were performed at room temperature for 30 min and the reaction products were resolved by electrophoresis on 4% polyacrylamide gels in 0.5 $\times$ TBE (45 m*M* Tris–HCl, pH 8.3, 45 m*M* borate, 2 m*M* EDTA) at 200 V at 4°.

UV cross-linking assay

UV cross-linking analysis was performed essentially as previously described (Wu, 1987). To prepare the DNA probe, the oligonucleotide 5′-AACGGGGTAAgctctCAGG-3′ was used as a primer and annealed to the upper strand of LSR-GBm (Fig. 3A). The filling-in reaction was then performed at 16° for 1 hr with 8 units of Klenow enzyme (Boehringer Mannheim) and 50 μ*M* each of dATP, dGTP, and Br-dUTP and 100 μCi of [α-^{32}P]dCTP. The binding reaction was performed as described above for EMSA except for using 200,000 cpm for the probe. The reaction mixture was then irradiated for 10 min with 254 nm UV light from a distance of 10 cm. The mixture was brought up to 25 m*M* $CaCl_2$ and 5 m*M* $MgCl_2$, and DNA was digested with 3.75 units of DNase I (Pharmacia) and 1 unit of micrococcal nuclease (Pharmacia) at 37° for 20 min and mixed with an equal volume of the sample buffer (50 m*M* Tris–HCl, pH 7.5, 5 m*M* EDTA, 8.8% SDS, 1.43 *M* β-mercaptoethanol, 22% glycerol, and 0.055% bromophenol blue). After heat denaturation at 100° for 5 min, the samples were separated on a 7.5% SDS–polyacrylamide gel.

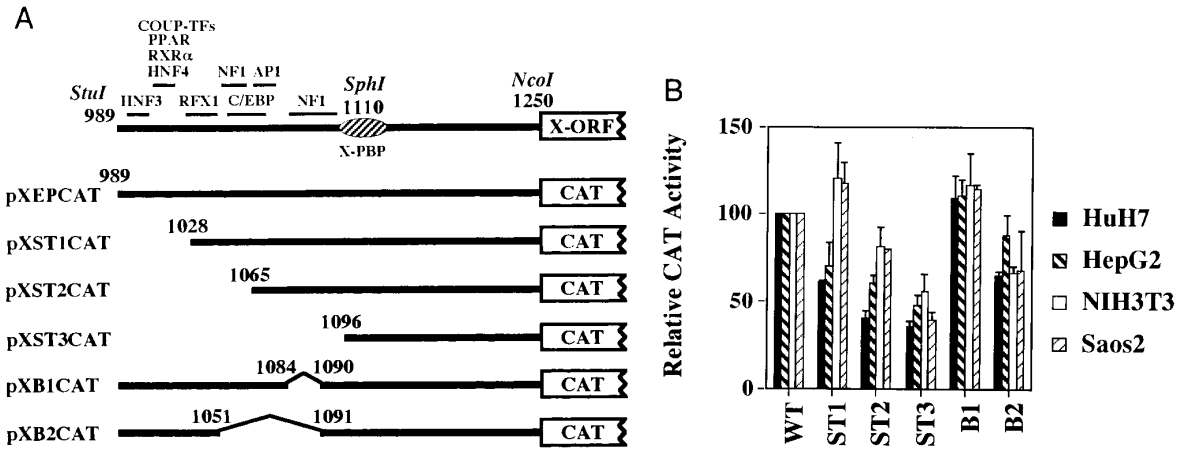

FIG. 1. Deletion analysis of the HBV enhancer I activity in the enhancer I/X gene promoter complex. (A) A schematic diagram of CAT plasmid constructs. Binding sites for the protein factors are shown on the top. X-PBP represents X promoter binding protein (Nakamura and Koike, 1992). (B) Activity assay for various CAT plasmid constructs transiently transfected into HuH7, HepG2, Saos2, and NIH3T3 cells. The extent of acetylation using various constructs was determined relative to that of pXEPCAT following normalization by β-gal activity. Results presented are the mean of at least four independent experiments with standard deviation.

RESULTS

Assignment of a specific region of enhancer I for the efficient transcription of X gene in the hepatic cell

Although several transcription factors could bind to the enhancer I region of HBV DNA, it is not well understood which factors play a major role in the efficient transcription of X gene in the hepatic cell. To assess a specific transcriptional regulation of X gene with respect to enhancer I (upstream of X-PBP), the HBV DNA fragment, containing enhancer I and X promoter regions, was cloned immediately upstream of the CAT reporter gene (Fig. 1A). A set of deletion mutants in enhancer I was then constructed and transfected into the human hepatoma cells (HuH7), the hepatoblastoma cells (HepG2), the human osteosarcoma cells (Saos2), and NIH3T3 cells. After normalization by β-gal activity, CAT activity of each deletion mutant was measured. A relative CAT activity to the level of expression derived from the wild-type plasmid, pXEPCAT, in each cell line is presented in Fig. 1B. Regarding the relative CAT activity of pXEPCAT in each cell line, CAT conversion rates were about 20–30% in HuH7 cells, 5–10% in HepG2 cells, and 1–2% in Saos2 and NIH3T3 cells under our experimental conditions.

Successive deletions progressing from the *Stu*I site showed that the deletion mutant of the LSR element (nt 989–1027) (pXST1CAT) reduced enhancer activity to 61% in HuH7 and 70% in HepG2, whereas in Saos2 and NIH3T3 cells this mutant exhibited no reduction of CAT activity compared with the wild-type (pXEPCAT). Additional deletion of the sequences between nt 1028 and 1064 (pXST2CAT) resulted in a reduction of CAT expression in all the cell lines examined. Further deletion of the sequences between nt 1065 and 1095 (pXST3CAT) resulted in an approximately 10% reduction of enhancer activity in HepG2, 25% reduction in NIH3T3, and 40% reduction in Saos2 compared with pXST2CAT, whereas the reduction of CAT activity in HuH7 cells was limited. Deletion progressing from the *Bal*I site showed that the region (nt 1085–1089) containing the NF1 binding site (pXB1CAT) was not significantly involved in the enhancer activity in all the cell lines used. Deletion of the sequences between nt 1084 and 1052 (pXB2CAT) resulted in an approximately 35% reduction of CAT activity relative to the wild-type in HuH7, NIH3T3, and Saos2 cells, and 15% reduction in HepG2 cells. These results indicate that the LSR element may be implicated in the specific activity of enhancer I for the X gene expression in the hepatic cell and that the remaining region of enhancer I contributed to full enhancer activity.

Hepatocyte-enriched transcription factors, HNF3, HNF4, and RXRα bind to the LSR element

EMSA was performed using the LSR element as a probe to detect protein factors involved in the specific function of enhancer I in the hepatic cell. It was recently reported that *in vitro* synthesized HNF3 could bind to the 2C/TGT3b (2C) site (nt 994–1007) (Chen *et al.,* 1994; Ori and Shaul, 1995) and that members of steroid hormon receptor superfamily, such as HNF4, RXRα, PPAR, and COUP-TFs, could bind to the GB/RARE (GB) site (nt 1010–1022) *in vitro* (Siddiqui, 1995). Since the LSR element contains these binding sites, we investigated whether these transcription factors bind to the LSR element in HuH7 cells by means of the competition assay. For the assay, oligonucleotides corresponding to each transcription factor binding sequence were prepared (Fig. 2A). Three specific retarded bands were identified when the nuclear extract from HuH7 was used (Fig. 2B). Bands A

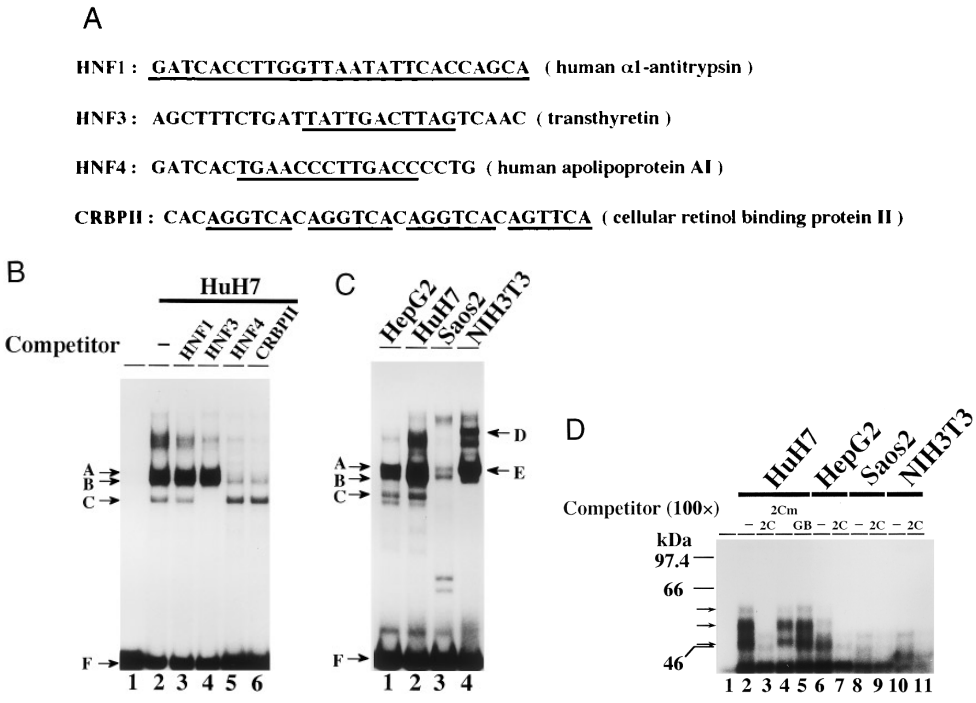

FIG. 2. Analysis of protein factors binding to the LSR element of enhancer I. (A) Sequences of oligonucleotides used in protein binding assays. Consensus binding sequences of each protein factor are underlined. Note: Each oligonucleotide is represented as a single-strand. (B) An electrophoretic mobility shift assay using a ^{32}P-labeled LSR DNA probe and 5 μg of HuH7 cell nuclear extract in the presence of 100-fold molar excess of the indicated double-stranded oligonucleotide. Arrows indicate the location of the specific DNA–protein complexes (A–C) and free probe (F). Nuclear extract was omitted from lane 1. (C) An electrophoretic mobility shift assay using a ^{32}P-labeled LSR DNA probe and 5 μg of nuclear extract from the indicated cells. (D) UV cross-linking analysis of the protein binding to the 2C/TGT3b site. UV cross-linking assay using ^{32}P-labeled LSR-GBm oligonucleotides and nuclear extracts from the indicated cell lines was performed as described under Materials and Methods. Arrows indicate the location of the specific DNA–protein complexes. Competitor indicates the presence or absence (–) of 100-fold molar excess of the indicated double-stranded oligonucleotide. The size of protein molecular weight markers in kilodaltons are indicated at the left. Nuclear extract was omitted from lane 1.

and B competed with the oligonucleotide CRBPII, representing the high-affinity RXRα, COUP-TFs, or PPAR-binding site from the promoter of the cellular retinol binding protein II gene (Mangelsdorf *et al.,* 1991; Kliewer *et al.,* 1992a, 1992b), and also by the oligo HNF4, representing the high-affinity HNF4-binding site from the promoter of the apolipoprotein AI gene (Fuernkranz *et al.,* 1994). The band C competed with the oligo HNF3, representing the high-affinity HNF3-binding site from the transthyretin promoter (Lai *et al.,* 1990), whereas no change of the shifted bands was observed by competition with the oligo HNF1, containing the well-characterized HNF1-binding site from the human α1-antitrypsin promoter (Monaci *et al.,* 1988) (Fig. 2B).

We also performed EMSA using nuclear extracts from four different cell lines to examine whether transcription factors interacting with the LSR element are diverse among these cell lines. The band C corresponding to HNF3 was detected with HepG2 cell nuclear extract as well as HuH7, but not with Saos2 or NIH3T3 cell nuclear extracts (Fig. 2C). Bands A and B, corresponding to members of steroid hormon receptor superfamily, were detected with nuclear extracts from all the cell lines examined, even though the amount of complex A (E) was more abundant than that of complex B when the NIH3T3 cell nuclear extract was used. In addition, a more slowly migrated band D was identified with NIH3T3 cell extract. It is noted that the quality of each nuclear extract was verified by the quantification of the contents of ubiquitous factors, NF1 and AP1, in each extract by virtue of EMSA using specific probes (data not shown).

The factor(s) binding to the 2C/TGT3b site was further analyzed by the UV cross-linking assay. Electrophoretic analysis of the samples on a 7.5% SDS–polyacrylamide gel revealed that the factor may be composed of three different proteins, whose molecular masses were estimated 58, 52, and 47 kDa (Fig. 2D).

Effect of point mutation within the LSR element of enhancer I on the X gene expression

To further assess the role of the LSR element in the transcriptional regulation of X gene, the mutant plasmids, each containing a point mutation within the LSR element were generated from the parental plasmid pXEPCAT by means of the PCR method, as depicted in Fig. 3A. Each mutant was confirmed by DNA sequencing and its capability of interacting with corresponding fac-

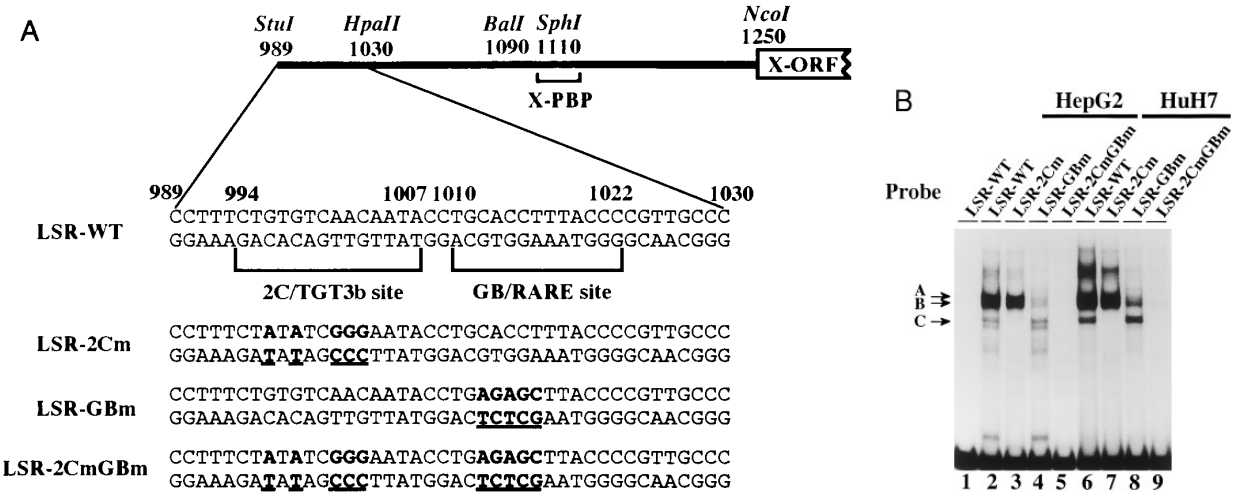

FIG. 3. The effect of point mutations within the LSR element on its protein binding capability. (A) Sequences of the wild-type and point mutant of the LSR element of enhancer I. (B) Mobility shift assay. The probes as indicated were incubated with nuclear extract from HepG2 or HuH7 cells. Arrows indicate the locations of the specific DNA–protein complexes. Nuclear extract was omitted from lane 1.

tors was analyzed by EMSA using nuclear extracts from HepG2 and HuH7 cells (Fig. 3B). Consistent to the previous results in Fig. 2C, the band C corresponding to HNF3 was detected with both nuclear extracts, when LSR-WT was used as the probe, but not when mutant LSR-2Cm was used. Bands, A and B, corresponding to members of steroid hormon receptor superfamily, were detected with both nuclear extracts, when wild-type LSR-WT was used as the probe, but much lesser extent when mutant LSE-GBm was used. No clear band was observed in case of double mutant LSR-2CmGBm as the probe. In transient transfection assays, the point mutation in the 2C/TGT3b site (pXEP2CmCAT) reduced the level of CAT activity to 58% in HuH7 and 74% in HepG2 cells. The mutation in the GB/RARE site (pXEPGBmCAT) also reduced to 39% in HuH7 and 49% in HepG2, while none of these mutations clearly affected the level of CAT activity in Saos2 and NIH3T3 cells (Fig. 4). These findings again indicate that the LSR element of enhancer I plays an important role in the efficient transcription of HBV X gene in the hepatic cell.

LSR element activates the transcriptional initiation from the X gene promoter M4

To generate the vector which efficiently expresses X gene in the hepatic cell, we introduced the LSR element into the pXM4CAT reporter, which contains only X gene promoter region (M4, nt 1102–1141) in the pSV00CAT vector to initiate the CAT gene transcription (Fig. 5). The LSR mutant vectors were also constructed. These expression plasmids were transfected into each cell line and CAT activities were measured, following normalization by β-gal activity. The pXM4-LSR2RCAT, which contains two copies of the LSR element in an inverted orientation, increased the level of CAT activity by about 10-fold compared with that of enhancer-less pXM4CAT in HuH7 cells (Fig. 5). Three copies of the LSR element

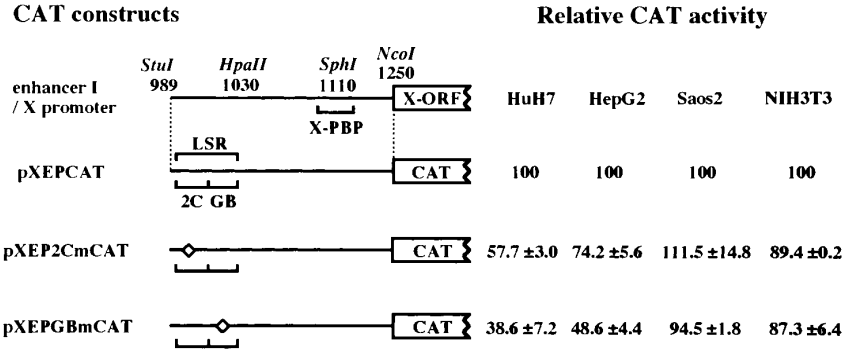

FIG. 4. Contribution of the LSR element of enhancer I to the expression of X gene in the context of the enhancer I/X promoter complex. A schematic diagram of CAT constructs used is shown on the left. The point mutation in the LSR element was introduced into the pXEPCAT. Diamonds indicate the mutation sites. Activities of various CAT plasmid constructs transiently transfected into HuH7, HepG2, Saos2, and NIH3T3 cells are shown on the right. The extent of acetylation using various constructs was determined relative to that of pXEPCAT following normalization by β-gal activity. Results are presented as the mean of at least four independent experiments with standard deviation.

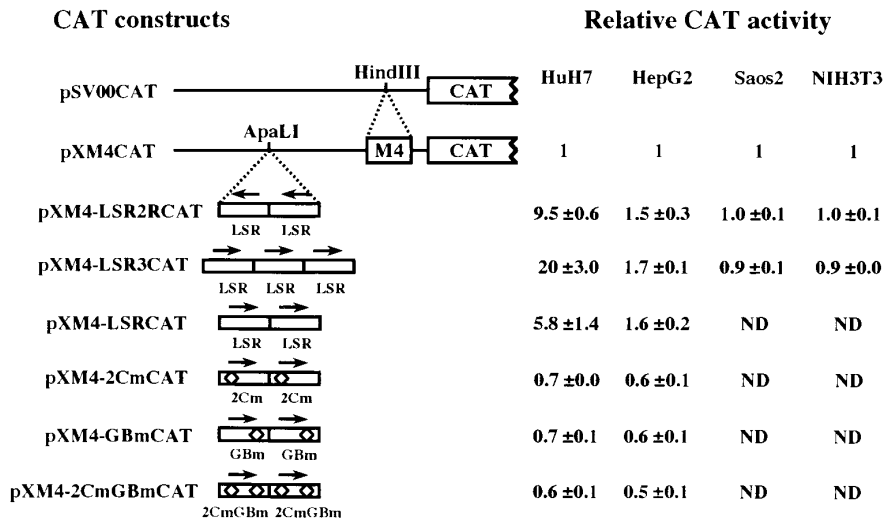

FIG. 5. Enhancer activity of the multimerized LSR element. The multimerized LSR element was introduced into the pXM4CAT which only contains the X gene basal promoter M4 to initiate the CAT gene transcription. 2Cm, GBm, or 2CmGBm represents the point mutant within the 2C/TGT3b, GB/RARE, or both sites, respectively. The arrows indicate the relative orientation of each fragment to the direction of transcription. Activities of various CAT plasmid constructs transiently transfected into HuH7, HepG2, Saos2, and NIH3T3 cells are shown on the right. The extent of acetylation in various reactions was determined relative to that of pXM4CAT following normalization by β-gal activity. Results are presented as the mean of at least four independent experiments with standard deviation. ND, not determined.

activated transcription from X gene promoter M4 by approximately 20-fold in HuH7 cells (pXM4-LSR3CAT). These constructs exhibited a slight increase of CAT activity in HepG2 cells (Fig. 5). An exact reason why HepG2 cell line responded in a lesser extent to these constructs than HuH7 cell line is not known. This transcriptional activation was not due to nonspecific effect, because the CAT activity of the LSR mutants remained at the basal level. Interestingly, none of these constructs containing the LSR element showed enhanced levels of CAT activity in Saos2 or NIH3T3 cells compared with that of the pXM4CAT (Fig. 5). These results clearly indicate that the LSR element can activate transcription from X gene promoter M4 efficiently in the hepatic cell in an orientation-independent manner.

DISCUSSION

HBV genome replication and gene expression in the hepatic cell are known to be facilitated by enhancer I and previous studies have shown that enhancer I exhibited a preference for the hepatic cell and mostly affected the activity of the core/pregenomic and X promoters (Shaul *et al.,* 1985; Jameel and Siddiqui, 1986; Roossinck *et al.,* 1986; Antonucci and Rutter, 1989; Lopez-Cabrera *et al.,* 1990). Although several binding sites for transcription factors including both liver-enriched and ubiquitous factors have been identified in enhancer I, a specific determinant for the X gene expression in the hepatic cell has not yet been well analyzed, especially in the context of the enhancer I/X promoter complex. In this study we elucidated the LSR element of enhancer I mainly involved in the efficient transcription of X gene in the hepatic cell. The deletion of LSR element reduced the activity of enhancer I in hepatic cells, but not in nonhepatic cells (Fig. 1). Based on the previous reports, the LSR element contains two protein binding sites; 2C/TGT3b site interacting with HNF3 (Chen *et al.,* 1994; Ori and Shaul, 1995), and GB/RARE site interacting with members of steroid hormon receptor superfamily (Huan and Siddiqui, 1992; Garcia *et al.,* 1993; Huan *et al.,* 1995). The point mutation in either site resulted in substantial diminution of the transcriptional activity of enhancer I in hepatic cells, but not in nonhepatic cells (Fig. 4).

The HNF3 family is composed of three isoforms (HNF3α, HNF3β, and HNF3γ) (Lai *et al.,* 1991), all of which are expressed mainly in the liver and gut and participate in the activation of liver-specific gene transcription by recognizing their target sequences as monomers. Those HNF3 binding sites have been described in the regulatory regions of the albumin (Jackson *et al.,* 1993), transthyretin (TTR) (Costa *et al.,* 1989), transferrin (Auge-Gouillou *et al.,* 1993), phosphoenolpyruvate carboxykinase (PEPCK) (Ip *et al.,* 1990), aldolase B (Raymondjean *et al.,* 1991) genes, and in duck hepatitis B virus enhancer (Liu *et al.,* 1994), as well. Electrophoretic mobility shift assays revealed that HNF3 factors likely exist in the nuclear extracts of HuH7 and HepG2, but not in Saos2 nor NIH3T3, and bound to the HS site of enhancer I (Fig. 2). According to the intensity of the signals, however, the amount of HNF3 factors in HepG2 may be less than those in HuH7 (Fig. 2C). In addition, the result of UV cross-linking analysis suggested that three isoforms of the HNF3 family might be expressed in HuH7, but only two isoforms in HepG2, although we could not

achieve further characterization of these factors in this inquiry. Along with the results of mutation analysis, our findings imply that HNF3 may be implicated in the transcriptional activation of HBV gene via its interaction with the 2C/GB site of enhancer I.

It is known that RXRα and HNF4 are expressed at higher levels in liver tissue than in other tissues (Sladek *et al.,* 1990; Mangelsdorf *et al.,* 1992) and can bind as homodimers to a DR1 element and function as strong constitutive transcriptional activators (Jiang *et al.,* 1995). PPAR is also a liver-enriched nuclear receptor and can bind as a heterodimer with RXRα to a DR1 and function as a transcriptional activator (Kliewer *et al.,* 1992b). On the other hand, COUP-TFs are ubiquitous factors and can bind as a heterodimer with RXRα to a DR1 and function as potent dominant repressors of both basal transcription and transactivation by RXRα (Kliewer *et al.,* 1992a; Ben-Shushan *et al.,* 1995). On the basis of this knowledge, HNF4, PPAR, and RXRα could function as transcriptional activators for HBV X gene in the hepatic cell by overcoming the repression activity of COUP-TFs, while the transcriptional activity of the GB/RARE site might be suppressed by COUP-TFs in nonhepatic cells, in which the expression of HNF4, PPAR, or RXRα is extremely limited. Taken together, both of the factors binding to the 2C/TGT3b and GB/RARE sites of enhancer I are very likely to contribute to efficient transcription of HBV X gene in the hepatic cell.

In regard to the remaining part of enhancer I other than the LSR element, many transcriptional factors have been demonstrated to bind to their respective binding sites (Ben-Levy *et al.,* 1989; Ostapchuk *et al.,* 1989; Patel *et al.,* 1989; Dikstein *et al.,* 1990; Faktor *et al.,* 1990; Lopez-Cabrera *et al.,* 1990; Trujillo *et al.,* 1991; Ori *et al.,* 1994). A few liver-enriched factors, e.g., C/EBP, are included in these factors, however, most of them are ubiquitous factors, e.g., RFX1, AP1, NF1. In fact, deletion analysis revealed that these sites are required for the maximal activity of enhancer I, but no marked specificity in the hepatic cell was observed (Fig. 1).

The role of the LSR element in the efficient transcription of X gene in the hepatic cell was also confirmed by transient transfection experiments using pXM4-LSRCAT (Fig. 5). This expression vector contains the X promoter M4 fragment and the LSR element as an enhancer for the CAT gene. We have previously demonstrated that the M4 fragment separated from the upstream region of X gene ORF possessed basal promoter activity (Takada *et al.,* 1996). The results from the CAT activity assay indicated that the LSR element stimulated the transcriptional activity of M4 promoter dramatically and exclusively in hepatic cells, especially in HuH7, but no activation was observed in nonhepatic cells. The low enhancer activity of the LSR element in HepG2 cells compared with that in HuH7 cells may represent the results that the activity of enhancer I in HuH7 cells from the differentiated hepatocellular carcinoma tissue in origin was higher than in HepG2 cells from the hepatoblastoma. In fact, we have previously demonstrated that the efficiency of HBV gene transcription was higher in HuH7 cells than in HepG2 cells, when pHBV-2 DNA (about 1.3 genome in size) was transfected into these two cell lines (Yaginuma *et al.,* 1987). It is further noted that this activation was completely obliterated by mutation in either the 2C/TGT3b or GB/RARE site alone. Data suggest that both factors binding to 2C/TGT3b and GB/RARE sites might be required to activate transcriptional regulation of X gene promoter. Since mutation in either site (2C/TGT3b or GB/RARE site) did not affect the factors binding activity of the other site in the electrophoretic mobility shift assay (data not shown), interaction between these factors is not likely to occur at least *in vitro.* One possible explanation is that this transcriptional activation may be due to stabilization of the basal transcriptional factor complex by both factors, as reported previously (Sauer *et al.,* 1995). To know whether or not these factors function as cooperative *trans*-acting factors on the X gene promoter, further investigation is required.

In conclusion, we elucidated that the LSR element of enhancer I plays a major role in the efficient transcription of the HBV X gene in the hepatic cell in the context of enhancer I/X promoter complex. It was also suggested that hepatocyte-enriched transcription factors, HNF3, HNF4, and RXRα are very likely to facilitate the efficient transcription from the X gene promoter via their interaction with the LSR element in the hepatic cell.

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