Expression and Secretion of Hepatitis B Viral Mutant Core Antigen

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B형 간염 바이러스의 돌연변이 내면항원의 발현 및 분비

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ABSTRACT : To study the role of mutant precore region in expression and secretion of hapatitis B viral core antigen, we have cloned core antigen gene(HBc) with or without precore region in heterologous expression vectors containing SV40 promoter, yeast promoter, and lambda P_L promoter. In COS cells transfected with plasmid containing C−gene with precore region, antigens were detected in both cell extract and cultured medium. However, in the cells transfected with plasmids containing C−gene without precore or with mutated precore region by one nucleotide (T) addition at the nucleotide 1,821, HBcAg was detected only in cell extracts. These results support that the mutation by one nucleotide addition shifted the initiation codon of precore region to 53 nucleotides upward and the elongated precore region also played a major role in the secretion of HBcAg in mammalian cells. In the case of yeast and *E. coli*, HBcAg was detected only in cell extracts in spite of the presence of precore region, which suggest that precore region could not affect HBcAg secretion in these system.

KEY WORDS □ Mutant HBcAg Gene, Expression, Secretion

Hepatitis B virus is a small human DNA virus which causes polymorphic liver diseases in human beings(Ganem and Varmus, 1987). Because hepatitis B virus was a major public health problem, many studies of HBV were concentrated on the production of hepatitis B vaccine using genetic engineering technique(Burrel *et al.*, 1981 ; Valenzuela *et al.*, 1982 ; McAleer *et al.*, 1984). But the biological studies of HBV life cycle and regulation of gene expression were hampered by the inability of virus propagation *in virto*. Nevertheless, cloning of HBV genome on *E. coli* has allowed the purification of genetically homogeneous HBV DNA and thus could make nucleotide sequence to be determined(Galibert *et al.*, 1979 ; Pasek *et al.* 1979 ; Valenzuela *et al.*, 1980 ; Fujiyama *et al.*, 1983 ; Ono *et al.*, 1983). Among the four open reading frames of the HBV(−) transcript, the C−gene encodes the core protein in *E. coli*(Pasek *et al.*, 1979 ; Edman *et al.*, 1981), in yeast (Kniskern *et al.*, 1986 ; Miyanohara *et al.*, 1986). Several studies in rodent cells transformed with HBV DNA(Gough and Murray, 1982 ; Gough, 1983 ; Manuel and Hirschman, 1984)and cloned core antigen gene(Ou *et al.*, 1986 ; McLachlan *et al.*, 1987 ; Roossinck and Siddiqui, 1987) were presented, but the mechanisms of core antigen gene expression and regulation were not clearly understood(Ganem and Varmus, 1987).

We have cloned HBV genome from a Korean patient previously(Choi *et al.*, 1984)and have determined the whole nucleotide suquence(Kim *et al.*, 1985 ; Rho *et al.*, 1989). The nucleotide suquence data revealed two in−phase initiation codon, but the initiation codon of precore region was found at upstream region of original initiation codon(1,758 position). Therefore, it was necessary to elucidate the effect of this elongated precore region on the expresion of core antigen by dissecting these three initiation codon respectively. For this purpose, we constructed several deletion mutants of core antigen gene with or without precore region by using Bal31 nuclease. We also studied the expression and secretion of core antigen in heterologous host systems.

MATERIALS AND METHODS

Chemicals

($\alpha-32p$)dATP(3,000 Ci/mmol) and($\gamma-32p$) ATP (2,900 Ci/mmol) were purchased from New England Nuclear(NEN). Sequencing reagents including dideoxynucleotide were obtained from New England Biolabs(NEB). Nitrocellulose filter(BA-85) was purchased from Schleicher and Schull(S & S). All other chmicals used were reagent grade.

Bacterial Strains

E. coli HB101 (F^-, r^-, m, recAB) was used for the isolation of plasmid and used as recipient of transformation. The host for transfection with M13 phage and isolation of phage DNA was *E. coli* JM107(△ Lacpro, F'traD36, $lacI^g$ Z △M15, endA, hsdR4). Cells were grown at 37℃ in LB or YT medium(5g of yeast extract, 8g of bactotryptone and 5g of NaCl per liter).

DNAs and Enzymes

All plasmid DNAs were isolated by the procedures of Birnboim and Doly(1979) and further purified by EtBr-CsCl density gradient ultracentrifugation for the transfection experiment(Maniatis *et al.*, 1982). BamHI linker(NEB 1017) was purchased from NEB. Restriction endonucleases were purchased from NEB or KOSCO. Other enzymes including T4 polynucleotidyl kinase, T4 DNA ligase Klenow fragment of *E. coli* DNA polymerase I and Ba131 nuclease were obtained from NEB. AMV reverse transcriptase was purchased from Life Science.

Enzyme reactions and DNA manipulation Techniques

Ligations were carred out according to published methods(Maniatis *et al.*, 1982; Song *et al.*, 1985). And for the end-labelling of oligonucleotide using ($\gamma-32p$)ATP, the reactions were heated at 90℃ for 2 min and unreacted ATP and inorganic phosphate were seperated from the labelled primer by Sephadex G-50(superfine) column(glass-bored 10ml pyrex pipette) pre-equiliberated with the buffer containing 50 mM triethyl ammonium bicarbonate (pH 7.8). All other reactions and transformation procedures were performed according to Maniatis *et al.*, (1982)

Determination of nucleotide sequence

The dideoxy sequencing reactions(Sanger *et al.*, 1977) using M13 phage template were carried out as described previosly(Kim *et al.*, 1985)

Expression of core antigen in mammalian cells

Transient expression of exogeneous DNA in mammalian cells was performed using calcium phosphate techinnques (Spandidos and Wilkie, 1984; Wigler *et al.*, 1979). Cells were grown in DMEM containing 10% fetal calf serum. In general, adherent cells were seeded into 25 cm^2 culture vessels 24 h prior to transfection at a density of 5×10^5 cells per plate. Ten microgram of plasmid DNA was mixed with sonicated calf thymus DNA to 20μg for each transfection. After 72 h, cells and cultured media were collected seperately.

Expression of core antigen in yeast and E. coli

Yeast strain SHY4(a, ura3-52, trp1-289, leu2-3, leu2-112, his3-1) was used as host for yeast transformation and the expression of core antigen. Yeast transformation was performed as described previously(Ito *et al.*, 1983). For the expression of core antigen in SHY4 strain harboring plasmid, the cells were cultured at 30℃, and harvested when the cell density reached to 1.1 at A^{550}. In the case of *E. coli*, temperature induction of core antigen was carried out by the procedures of Mott *et al.* (1985)

Radioimunoassay

The Abbott-HBe kit(Abbott Laboratories) was used for measuring HBcAg and HBeAg. And assays were carride out by manufacturer's guideline. Detection of antigens by slot-blot immunobinding assay with S & S minifold II (Schleicher and Schull) was performed by standard procedures as described previously(Jahn *et al.*, 1984).

Mapping of DNA end deleted with Bal31 nuclease

Plasmids were codigested with BamH I and Bgl II, phenol/chloroform treated, precipitated with ethanol and resuspended in gap filling buffer. Polymerization reactions were performed using($\alpha-32p$) dATP (5μCi) and Klenow fragment of *E. coli* DNA polymerase I at 30℃ for 15 min, chased with 1mM(final) of dNTP for another 15 min reaction, stopped with sample buffer and analysed with denaturing sequencing gel. Sizes were determined after autoradiography comparing with size marker (M13mp9 sequencing ladder).

RESULTS

Constrution of recombinant plasmids

For the expression of core antigen containing precore region, pSVHBcP plasmid was constructed (Fig. 1). The core antigen gene was obtained from the Hinc II digestion of pHBVD107(Kim *et al.*, 1985), which contained head to tail dimer form of HBV DNA. After cloning of Hinc II fragment(0.9kb) into the multiple cloning site of pBLU vector (Bluescribe vector of Vector Cloning Systems), the recombinant plasmid pHBcR was selected by the restriction endonuclease digstion. For the expression of core antigen gene in mammalian cells, eukaryotic

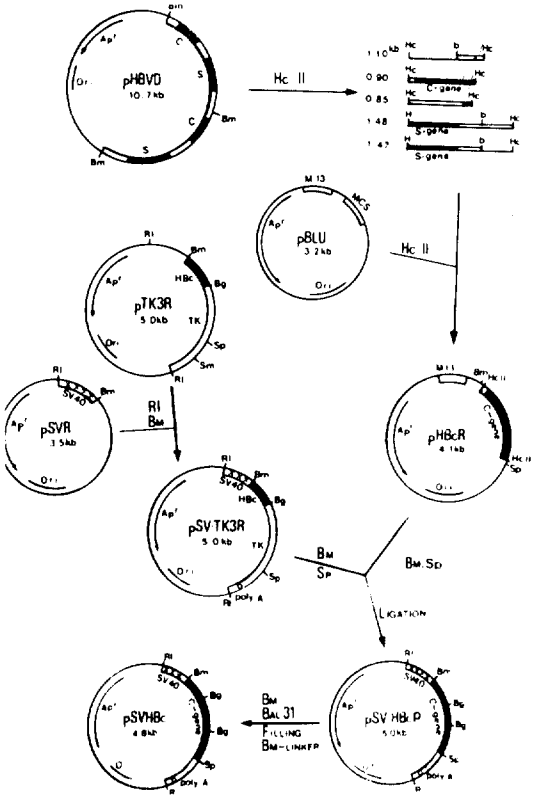

Fig. 1. *Construction Strategy of Recombinant Plasmids.* Abbreviations : HBc and C, core antigen gene;Ap^r, ampicillin resistence ; s, surface antigen gene ; TK, thymidine kinase gene of HSV ; ori, origin of replication ; SV40, SV40 replicon and promoter region ; M13, M13 phage replicon ; MCS, multiple cloning sites ; poly A, poly(A) addition signal sequence ; Hc or HcII, HincII ; b or Bm, BamHI ; Sp, SphI ; Bg, BglII ; RI, EcoRI ; Sm, SmaI ; kb, kilo base ; pBLU, Bluescribe vector (obtained from Vector Cloning Systems).

expression vector pSVTK3R was constructed. This vector contained the SV40 promoter, replicon, and the structural gene of herpes virus thymidine kinase containing poly(A) addition signal. Therefore such vector is able to replicate multiple rounds in COS cells which produce SV40 large T antigen(Gluzman, 1981). By the insertion of BamH I −Sph I DNA fragment(0.9 kb DNA fragment generated by the digestion of pHBcR) into the pSVTK3R vector, the pSVHBcP recombinant plasmid was constructed.

For the expression of core antigen in yeast, the vector pBY7 was used which contained the promoter region and poly(A) addition signal of phosphoglycerate kinase gene. pGKHBcP was constructed by the insertion of EcoR I fragment(0.9 kb) of pHBcR2, which was derived from pHBcR by EcoR I linker insertion in the Hind III site of pHBcR, into the corresponding site of pBY7. *E. coli* vector pPLHBcP was constructed by the insertion of EcoR I fragment of pHBcR2 into the EcoR I site of pPL13 which contained P_L promoter of lambda phage and ribosome binding site. pGKHBc and pPLHBc which contained core antigen gene without precore region was constructed by the insertion of EcoR I fragment of pSVHBcR, a derivative of pSVHBc, made by EcoR I linker insertion in the BamH I site of pSVHBc.

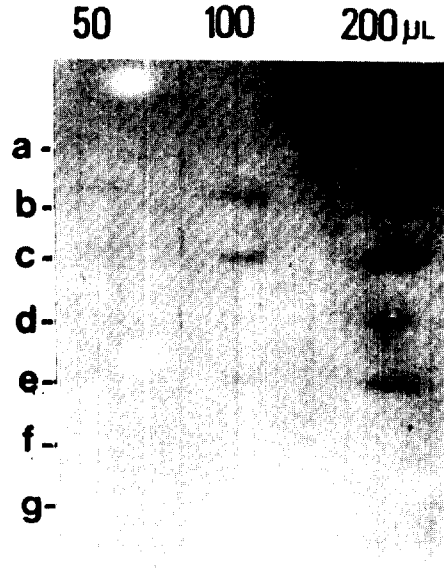

Fig. 2. *Identification of HBcAg and HBeAg by Immunobinding assay.*
Samples from the cells transfected with pSVHBcP (as indicated in the top of Figure) were slot blotted onto the nitrocellulose filter, and incubated with blocking solution containing 50 mM Tri-HCl, pH 7.4, 0.2 M NaCl(TN buffer and 5%(w/v) bovine serum albumin at 25℃ for 1 h. The paper was then incubated with ^{125}I-labelled antibody (2×10^6 cpm/ml) in TN buffer containing 0.1%(v/v) Triton X-100 at 25℃ for 5h, followed by washing and autoradiography. Lane a, mock infected COS cell extract ; Lane b and c represent COS cell extracts after 48h and 72h transfection, respectively ; Lane d and e represent culture medium after 48h and 72h transfection, respectively ; Lane f and g show the culture medium from HEPA-1 cells after 48h and 72h transfection, respectively.

Transient expression of core antigens in COS cells

In order to investigate the expression of core antigen, samples were assayed with Abbott−HBe kit which could detect both HBeAg and HBcAg (Petit and Pillot, 1985). Fig. 2 shows the identification of expressed antigens by direct immunobinding assay. COS cells transfected with pSVHBcP plasmid containing core antigen gene with elongated precore region expressed HBcAg and HBeAg(Fig. 2 lane b− e). Antigens were detected in both cell extracts and cultured medium(Fig. 2 lane d and e). These data demonstrated that the expressed antigens were secreted into the culture medium. The COS cells transfected with pSVHBcP plasmid expressed antigens continuously through 10 days, showed a maximum secretion ratio between second and third day(data not shown). In Hepa−1 cells (mouse hepatoma cell line), antigens were not detected by direct immunobinding assay(Fig. 2 lane f and g)

Influence of elongated precore region on the secretion of core antigen

To elucidate the role of elongated precore region in secretion of HBeAg in COS cells, we constructed various mutant plasmids including pSVHBcP4 and pSVHBc plasmid which contained core gene with frameshifted precore and without precore, respectively. For the construction of deleted recombinant plasmid, pSVHBcP was digested with BamH I followed by serial deletion with Bal31 nuclease. After gap−filling with Klenow fragmet, BamH I linker was added to determine the location of deleted 5' DNA end later. To recover the promoter region of SV40 which was deleted by the Bal31 digestion, SV40 promoter region of pSVTK3R(0.4 kb Hind Ⅲ−BamH I DNA fragment) was inserted in the corresponding sites of selected plasmids. The 5' ends of deletion mutants were determined as described in Materials and Methods(Fig. 3 and Fig. 4). Mutant plasmids were transfected into COS cells and expressed antigens were measured 3 days posttransfection. As shown in Fig. 4, the highest amount was detected in COS cells transfeted with pSVHBcP1 plasmid, whose 5' end was 180 bp apart from the initiation site of core antigen.

To study the role of precore region in the secretion of HBeAg, recombinant plasmid pSVHBc containing only core antigen was selected. In the case of pSVHBcP, antigens were deteced both cell extracts and cultured medium(Fig. 2 and Fig. 4). In the samples of COS cell transfected with pSVHBc, however, antigens were detected in cell extracts but not in the medium(Fig. 4). These results suggested that the precore region might play a critical

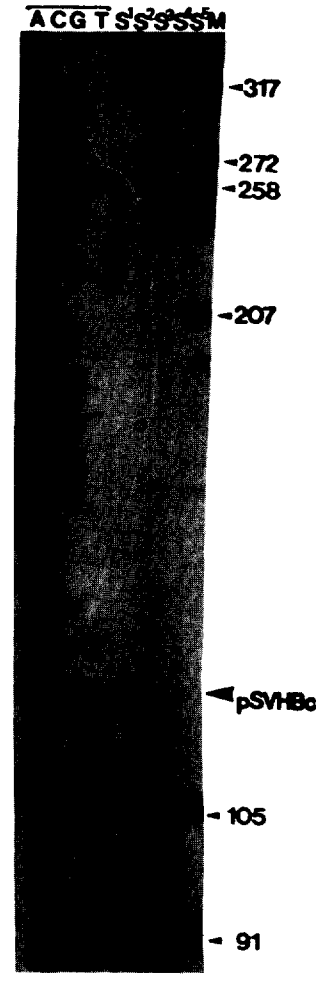

Fig. 3. *Mapping of the DNA End Deleted with Bal 31 Nuclease.*
Mapping of the DNA end deleted with Bal31 nuclease. End-labelling and sequencing reactions were carried out as described in Materials and Methods. Arrows (s1-s5) are end-labelled DNA bands originated from digestion with BamHI and BglII. Sequencing ladder were made by sequencing of M13mp9. Numbers are size of DNA marker generated by end-labelled pBR322 digested with Sau3A.

role in the secretion of HBeAg. This result was also confirmed by the facts that in COS cells transfected with pSVHBcP4, which contained core antigen gene with frameshifted precore by one nucleotide(T) addition, antigens also expressed, but the antigens were detected only in cell extracts(Fig. 5).

Table 1. *Expression of HBcAg and HBeAg in Yeast and E. coli*

fraction	cpm*					
	Yeast(SHY4)			*E. coli*(M5248)		
	mock	pGKHBcP	pGKHBc	mock	pPLHBcP	pPLHBc
Cell extract	252	1,225	21,945	463	129,300	178,9935
Cultured medium	292	515	468	420	421	405

* cpm of HBcAg and HBeAg 1×10^8 cells

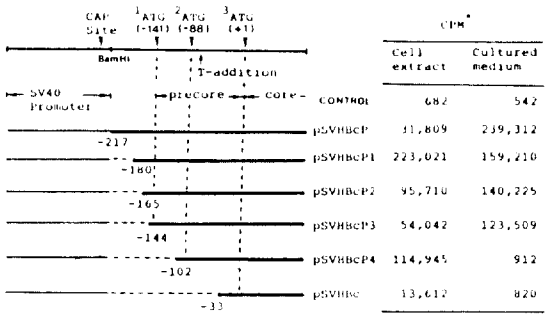

Fig. 4. *Schematic Diagram of 5'Deletion Mutants and Relative Value Represented as cpm of Core Antigen Expressed in COS cells.*
Three ATG codons are designated as number: 1, ATG codon for elongated precore; 2, ATG codon for mutated precore by one nucleotide(T) addition; 3, ATG codon for core antigen. Every end points of 5'deletion mutants and names of recombinant plasmids are shown in the middle region. Dotted lines indicated the region deleted with Bal31 nuclease. COS cells were transfected with each plasmid and produced antigens were assayed as described in Materials and Methods.
* cpm of HBcAg per 5×10^5 cells.

Expression of core antigen in heterologous host system To investigate the expression of core antigen and the role of elongated precore region in secretion of HBeAg other than mammalian system, we cloned the core antigen gene in yeast and *E. coli* expression vector(data not shown) and measured the production of antigens. In the case of yeast and *E. coli*(Table 1) antigens were not detected in culture medium in spite of the presence of precore region.

DISCUSSION

To study the expression of core antigen in COS cells and the role of precore region in the secretion of HBeAg, we subcloned core antigen gene with or without precore region into the mammalian expression vector pSVTK3R. COS cells transfected with one of the resultant recombinant plasmid pSVHBcP, which contained core antigen gene with elongated precore region expressed HBcAg and HBeAg efficiently. The antigen was also detected in the medium(Fig. 3). These secreted antigens seemed to be HBeAg alone(Ou *et al.*, 1986; McLachlan *et al.*, 1987). Because antigens secreted in the medium were detected continually during 10 days posttransfection(data not shown), it seemed that HBeAg might be a stable protein. The highest amounts of antigens were detected in the case of a deletion mutant that the 5' end was 180 bp apart from the intiation site of core antigen(pSVHBcP 1).

In the case of elongated precore region, the problem of ribosome binding site was emerged. Kozak(1986) proposed that the optimal context for initiation is CCPuCCAUGG(Pu; purine). But if the sequence around the first AUG triplet is suboptimal, some 40S subunits bypass that site and intiate translation in farther downstream. In consideration of that theory the optimal context could be occurred in the ATG codon of number 2(Fig. 4). The ATG codon of number 1 and number 3, however, could be also served as optimal context of initiation for the expression of core antigen since COS cells transfected with plasmids constructed in this study expressed antigen efficiently (Fig. 4). And it suggested that antigens detected from cytoplasm of COS cells transfected with pSVHBc and pSVHBcP4 plasmids should be originated from ATG number 3 in Fig. 4. Therefore, antigens could not be secreted into the medium in the absence of precore region (Fig. 4).

Because our nucleotide sequence data of core antigen coding region (Kim *et al.*, 1985; Rho *et al.*, 1989) showed one nucleotide(T) addition at nucleotide 1,821, the elongated precore region raised the problem whether precore region could have any role in HBV life cycle. We have compared the mode of expression in COS cells transfected with plasmids containing core antigen gene with or without elongated precore region. These results showed that the elongated precore region could facilitate the secretion of HBeAg into the medium in COS cells (Fig. 2 and Fig. 4), which coincided with the reports

of previous studies (Ou *et al.*, 1986 ; Roossinck *et al.*, 1986). This result was also confirmed from the COS cells transfected with pSVHBcP4 plasmid containing core gene with mutated precore region (Fig. 4). These results support that the mutation by one nucleotide (T) addition shifted the initiation codon of precore region to 53 nucleotides upward and the elongated precore region also played a critical role in the secretion of HBcAg in mammalian cells. Our results would be the first case using the mutated precore for elucidating the role of precore region.

It was suggested that the biogenesis of HBeAg could be resulted from proteolytic cleavage of core antigen (Takahashi *et al.*, 1983), and only HBeAg could be secreted in mammalian cells (Ou *et al.*, 1986 ; McLachlan *et al.*, 1987). Thus the cleavage of core antigen in carboxy terminus for HBeAg synthesis might also play a role in the secretion besides the cleavage in precore region by assumptive signal peptidase. Miller (1987) proposed that the proteolytic self-cleavage of HBV core antigen may generate serum HBeAg by showing the homology between the protease like sequence of hepadnaviruses and the protease sequence of retroviruses. The role of HBeAg in HBV life cycle was not concluded yet (Tiollais *et al.*, 1985 ; Ganem and Varmus, 1987). Whatever the role of HBeAg in HBV life cycle may be, it will be worth trying to elucidate the mechanism of cleavage reaction of core antigen.

In the case of yeast and *E. coli*, antigens were not detected in culture medium in spite of precore region (Table 1). These results indicated that elongated precore region could not affect the secretion of core antigen in these systems.

적 요

포유동물 세포내에서 간염 바이러스의 내면항원의 발현과 전위내면 항원(precore) 부위의 역할을 규명하기 위하여 고등동물세포 발현용 벡터에 전위내면항원 부위를 갖거나 또는 갖지 않는 내면항원 유전자를 클로닝 하여 COS 세포내에서의 발현을 조사하였다. 전위내면항원 부위를 포함한 내면항원 유전자를 갖는 플라스미드로 감염시킨 COS 세포는 항원들이 세포추출물과 배양액에서 검출되었다. 분비된 항원의 증가율은 감염후 2일과 3일 사이가 가장 높았고, 부분결실된 제조합 플라스미드중 내면항원의 ATG codon에서 180 bp 떨어진 것이 가장 발현이 잘 되었다. 전위내면항원을 갖지 않거나 하나의 염기가 첨가되어 변형된 전위내면항원을 갖는 제조합 플라스미드의 경우 항원들이 세포추출물에서만 검출 되었다. 이러한 사실은 전이내면항원 부위가 HBe 항원의 분비에 관여 한다는 사실을 의미한다. 그러나 대장균이나 효모 세포의 경우는 전위내면항원의 존재와 상관없이 항상 세포추출물에서만 존재하는 것으로 보아 이들 세포의 경우에서는 전위내면항원 부위가 HBe 항원의 분비에 영향을 줄 수 없음을 의미한다.

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