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Role of Pre-C Region in the Expression and Secretion of Hepatitis B Viral Core Antigen in Yeast

Shin, Sang-Hoon, Seong Kee Kim and Hyune Mo Rho
Laboratory of Molecular Genetics, Department of Zoology,
Seoul National University, Seoul 151-742, Korea

효모에서 B형 간염바이러스의 내면항원의 발현과 분비에 미치는 전위내면항원의 역할

신상훈·김성기·노현모
서울대학교 동물학과 분자유전학실험실

ABSTRACT: The coding sequence of hepatitis B viral core antigen (HBcAg) (subtype *adr*) contains two in-phase initiation codons, one for precore and the other for core antigen gene. To study the expression of core antigen and the role of precore region, the coding sequence of HBcAg with or without precore (pre-C) region were subcloned into yeast expression vector containing phosphoglycerate kinase (PGK) promoter. To study the role of upstream region in the expression of the core antigen, a series of 5' deletion mutants were also subcloned into the vector.

After transformation into various host strains, the expression of HBcAg were analysed by radioimmunoassay. Under optimal condition of core antigen gene expression in yeast, the highest amount of antigen was detected in the cell line SHY4 containing pGKHBc plasmid composed of the yeast PGK gene promoter, terminator and C-gene.

Regardless of the presence of precore region, core antigen was not detected in the medium but in cell extract. These results suggest that precore region cannot affect the secretion of core antigen in *Saccharomyces cerevisiae*.

KEY WORDS□HBcAg, Yeast PGK promoter

Hepatitis B virus (HBV) is a 3.2 Kb long, partially double-stranded circular DNA virus, which belong to hepadnaviridae. HBV causes serious liver disorders and is linked to later development of hepatoma. Despite its crucial role in human health problems, its narrow host range and inability to be propagated in cultured cells stymied early efforts to elucidate the molecular details of viral replication. However, advances in molecular genetics make it possible that the viral genome cloned and sequenced, and provide surrogate systems for the understanding of molecular mechanism of this virus replication (Tiollais *et al.*, 1981; Tiollais *et al.*, 1985). Although various subtypes of HBV genomes are cloned and sequenced, the nucleotide sequence showed considerable heterogeneity between subtypes and even within the same subtypes (Wain-Hobson and Tiollais, 1984). Previously, HBV genome from Korean patient was cloned (Choi *et al.*, 1984) and the whole nucleotide sequence of HBV genome (subtype *adr*) was determined in our laboratory (Kim *et al.*, 1985; Rho *et al.*, 1989). The analysis of sequence showed four open reading frames (ORF); S, C, X-C, P. The nucleotide sequence of the core open reading frame (C gene) reveals several interesting features. C-gene has two in-phase initiation codons separated by 141 base pairs (bp) in the same ORF. The polypeptide produced by translating from the upstream or downstream initiation codon consists of 230 or 183 amino acids, respectively. The 3'-end of C gene encodes an extremely

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basic, arginine-rich, protamine-like, segment that would be expected to confer nucleic acid (HBV genome) binding capacity on the protein.

HBeAg is a cryptic determinant released by disruption of the nucleocapsid (Ohori *et al.*, 1979), and is present in the serum as a protein of 15 K dalton which may result from proteolytic cleavage of major core protein P22 (Takahashi *et al.*, 1983). The C-terminal sequence (Thr-Thr-Val-Val) was determined for serum HBeAg. But the precise biogenesis of HBeAg has not yet been clearly elucidated. For these regions, the role of pre-C region in core antigen gene was correlated with HBeAg and secretion.

The efforts to produce core antigen in *E. coli* (Pasek *et al.*, 1979; Edman *et al.*, 1981), in mammalian cells transformed with HBV DNA were presented (Roossinck *et al.*, 1986; Manuel and Hirshman, 1984), but the mechanism of core antigen gene expression and regulation, however, were not clearly understood.

Yeast, especially strains of *Saccharomyces cerevisiae,* are important experimental organisms for mordern biological research, with good reasons. It was chosen for the extension of gene cloning work in *E. coli* to in eukaryotic organisms. In this study, the cloning of hepatitis B viral core antigen gene (*adr*) fragment in yeast expression vector including phosphoglycerate kinase gene promoter and expression of HBcAg in yeast were described.

MATERIALS AND METHODS

Bacterial strains and yeast strains

E. coli strain HB101 (F^-, r^-, m^-, recA13) was used for isolation of plasmid and as a host for transformation. The cells were grown at 37 °C in LB medium (10 g of Bacto-tryptone, 5 g of Yeast extract and 10 g of NaCl per liter). *Saccharomyces cerevisiae* strains used in this work are listed in Table 1. Yeast cells were grown at 30 °C in YPD medium (10g of Yeast extract, 20g of peptone and 20g of dextrose per liter).

DNAs

E. coli plasmid DNAs were isolated by the alkaline lysis method (Birnboim and Doly, 1982), and plasmids from yeast were prepared as the method described by Devenish *et al.* (1982). HBcAg gene containing deleted precore region was obtained by electroelution (Maniatis *et al.*, 1982) from pSVHBcP deletion series constructed previously. (Kimet *et al.*, 1989). Yeast expression vectors pBY7 (PGK promoter-terminator, Kim *et al.*, 1988) and pY15 (ADH promoter and FLP terminator in yeast 2 μm plasmid) were described previously.

Table 1. *Saccharomyces cerevisiae strains*

Strains*	mating type and genotype
SHY4	a ste-VC9, ura3-52, trp1-289, leu2-3, leu2-112, his3- Δ1
SL560-3A	a his3- Δ1, leu2-1, met8-1, trp1, ura3-52
YNN27	α trp1-289, ura3-52, gal2 (612)
D13-1A	a trp1, his3-532, gal2, suc2, cup1, mal^-
SC3	α trp1, ura3-52, his3, gal2, gal10/(cir°)
20B-12	α trp1, pep4-3

*Strains except SHY4 and SL560-3A were obtained from KAIST, Genetic Engineering Center, KCTC.

Enzymes and reagents

Restriction endonucleases were purchased from NEB, BRL and KOSCO. T4 DNA ligase, Klenow fragment of *E. coli* DNA polymerase I were purchased from NEB. Lyticase was purchased from Sigma. Media components were purchased from Difco. All other chemicals used were reagent grade.

DNA manipulation techniques

DNA manipulation and other cloning procedures were performed according to Maniatis *et al.* (1982).

Yeast strains were transformed to the method of Ito *et al.* (1983). Yeast transformants were selected on Burkholder minimal medium (Bostian *et al.*, 1980), and cultured in YNB + CAA medium (6.7 g of yeast nitrogen base without amino acids, 10 mg of adenine, 5 g of casamino acids and 20 g of glucose per liter).

Hepatitis B viral core antigen assay

Recombinant DNAs were used to transform yeast strains to tryptophan prototrophy. One yeast trp^+ transformant colony was picked and grown in 10 ml of YNB + CAA medium at 30 °C with aeration until A_{660} reached 1.0. The cells were collected and suspended in 3 ml of solution containing 100 μg/ml of Lyticase, 1.2 M Sorbitol, 10 mM KH_2PO_4 (pH 6.8) and incubated for 1 hr at 30 °C. The spheroplasts were centrifuged and resuspended in 500 μl of PBS buffer containing 1 mM PMSF. Sterile glassbeads (0.45-0.50 mm in diameter, from Sigma) were added until just below the level of the cell suspension, and vortexed to the highest for 30 min using ice bath to keep the mixture cool. The lysate was centrifuged and the supernatant was assayed by Abbott-HBe kit (Abbott Labs).

RESULTS AND DISCUSSION

Recombinant DNA construction of core antigen .

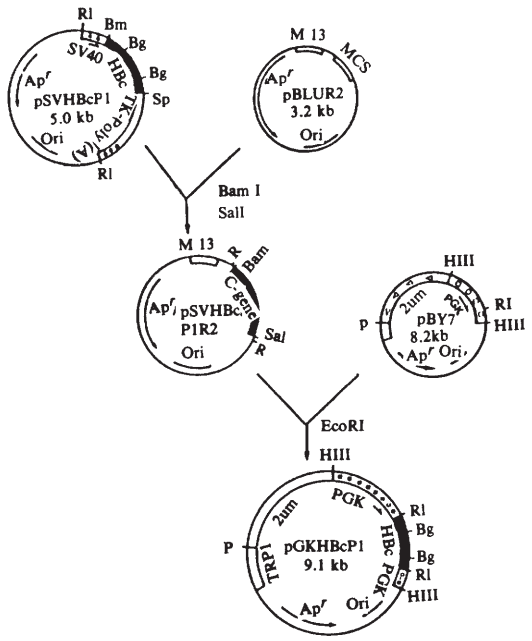

Fig. 1. *Construction strategy of recombinant plasmids.* Construction of all recombinant plasmids are described in the text. Abbreviations: HBc and C-gene, core antigen gene; ori, origin of replication; SV40, SV40 replicon and promoter region; TK, thymidine kinase gene of HSV; Poly (A), poly(A) addition signal sequence; TRP1, tryptophane marker gene for selection in yeast; 2 μm, yeast 2 μm plasmid fragment containing replicon; MCS, multiple cloning site; M13, M13 phage replicon; Ap^r, ampicillin resistant gene; PGK, phosphoglycerate kinase gene; ADH, alcohol dehydrogenase gene promoter; R or RI, EcoRI; P, PstI: Bm or Bam, BamHI; Bg, BglII; HIII, HindIII; Kb, kilo base.

with or without precore region for the expression in yeast

For the expression of core antigen gene containing pre-C region, various plasmids were constructed. Fig. 1 shows the typical cloning strategy as a representative. Core antigen gene containing pre-C region was obtained from pSVHBcP1 deletion series (Kim, *et al.*, 1989). First of all, pSVHBcP1 was digested with BamHI and SalI, and the fragment of C-gene was inserted into the multiple cloning site of pBLUR2 which contains two EcoRI sites at the both ends of multiple cloning site. The C-gene fragment obtained by EcoRI digestion of pSVHBcP1 R2 and by electroelution cloned into yeast expression vector pBY7 and pYJ5 to produce pGKHBcP1 and pAdhHBcP1, respectively. Other C-gene fragments containing various deletion ends were cloned through these same steps.

Table 2. *Expression of HBcAg in Yeast*

Host plasmid	cpm*			
	SHY4		SL560-3A	
	cell extract	culture medium	cell extract	culture medium
pGKHBcP3	1,406	436	809	456
pGCHBC	17,554	428	1,479	N.D.**
pAdhHBcP3	596	453	600	410
pAdhHBc	963	N.D.	632	N.D.

*cpm of negative control was 252 ± 5 (n = 3).
Cutoff value = 2.1 × 252 cpm = 529 cpm. Samples with cpm less than the cutoff value are considered nonreactive for HBeAg by the criteria of the Abbott-HBe test.
**N.D. = not determined.

The C-gene of the HBV genome contains two conserved in-phase initiation codons separated by 141 nucleotides (*adr*). The clones of C-gene containing precore region selected above had different end points generated by Bal31 digestion, that is, P, P1, P2, P3, P4, and C clones had end points of deletion at -217, -190, -165, -144, -102 and -33, respectively. When the downstream ATG codon of core antigen is numbered as +1, pSVHBcP3 has -3 end point from upstream precore initiation codon. Thus, P3 and C clones were chosen firstly to express core antigen with or without precore region in yeast SHY4 and SL50-3A.

Expression of core antigen in yeast

Constructed yeast expression vectors containing P3 and C clones were transformed into yeast. Expression of HBcAg and HBeAg in cell extracts and cultured medium were analyzed by radioimmunoassay with Abbott-HBe kit.

pGKHBc, which contains PGK promoter, C-gene and PGK terminator in SHY4, expressed highest level of HBcAg, and pGKHBcP3, which contains PGK promoter, C-gene containing precore region and PGK terminator in SHY4, expressed lower level of HBcAg in contrast to pGKHBc (Table 2). Regardless of the presence of precore region, core antigen was detected not in the medium but in cell extracts only. These results suggest that precore region cannot affect the secretion of core antigen in yeast.

Generally, SHY4 shows property of better host systems for HBcAg than SL560-3A. Vector containing ADH promoter FLP terminator and C-gene expressed less HBcAg than vector containing PGK promoter, terminator and C-gene. These difference of promoters caused the distance effect between regula-

Table 3. *Expression of HBcAg in Yeast*

host plasmids	SHY4	SL560-3A	YNN27	D13-1A	SC3	20B-12
			cell extracts (cpm)*			
pGKHBcP1	843	—**	853	—	—	—
pGKHBcP2	1,119	—	793	678	—	1,033
pGKHBcP3	1,406	809	779	746	766	1,349
pGKHBc	17,554	1,479	1,119	895	984	15,150
pAdhHBcP1	782	—	688	—	—	—
pAdhHBcP2	578	—	816	—	814	860
pAdhHBcP3	596	600	—	—	—	850
pGKHBc	963	632	—	—	—	856

*cpm of negative control was 252 ± 5 (n = 3).
Cutoff value = 2.1 × 252 cpm = 529 cpm. Samples with cpm less than the cutoff value are considered nonreactive for HBeAg by the criteria of the Abbott-HBe test.
**The bars (-) mean not determined.

tory sequence in yeast derived promoter and HBV derived C-gene containing clone. To overcome this factor in the expression of precore region, other clones containing P1 and P2 C-gene fragment were tested.

The expression of foreign genes was changed according to yeast strain types. It was suggested that the nature of this effect has not thoroughly examined but appears to be related to a decrease in plasmid stability compared to other strains (Hitzemann *et al.*, 1983). Thus other four additional strains (Table 1) containing 20B-12 which is proteinase A mutant strain were tested for the best expression strain.

Expression of precore region in yeast

Expression of precore region in several yeast strains containing various expression vectors were listed in Table 3. Strain effects and distance effects explained above were compared.

The expression of core antigen gene by pBY7 was so high that this phosphoglycerate kinase vector system is proved to be effective in the expression of foreign genes. But the relative low level expression of core antigen containing precore region region involves complicated problems, even various combination of the different deleted end clones and PGK and ADH promoter does not produce high levels. The core antigen with or without precore region was expressed in yeast but the antigens were not detected in the medium, suggesting that the precore region can not affect the secretion of the core antigen in yeast. All cpm value of sample in culture medium is less than the cutoff value, so they are not denoted in Table 3.

적 요

B형 간염바이러스(*adr* 형)의 내면항원(HBcAg) 유전자는 두개의 단백질 합성시작 유전자 암호 ATG를 갖는다. 하나는 전위내면항원을 다른 하나는 내면항원 유전자를 위한 ATG 부호이다. 내면항원의 발현과 전위내면항원의 역할을 연구하기 위하여 전위내면항원 유전자를 포함하는 것과 포함하지 않는 내면항원 유전자를 효모발현 운반체에 클로닝 하였다. 또한 내면항원의 발현에 5' upstream의 역할을 알아보기 위하여 여러 가지의 5' 제거돌연변이체를 클로닝하였다.

앞에서 만들어진 플라스미드로 여러 효모 균주를 형질전환시킨 후 발현된 내면항원과 그와 관련된 항원 HBeAg을 방사면역측정법으로 확인하였다. 효모에서 내면항원 발현의 최적조건 하에서 가장 높은 수준의 항원은 PGK promoter와 terminator에 내면항원을 포함한 pGKHBc를 가진 SHY4에서 검출되었다.

전위내면부위의 존재와 무관하게 내면항원은 배양액에서는 검출되지 않고 세포내에서만 검출되었다. 이 결과는 전위내면항원이 효모내에서 내면항원의 분비에 영향을 주지않음을 의미한다.

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