KOR. JOUR. MICROBIOL. May, 1991 p. 80-84 Vol. 29, No. 2

Expression of Hepatitis B Viral Core Antigen Gene in *Escherichia coli*

Choi, Soo Keun, Won Sang Lee, Seong Kee Kim and Hyune Mo Rho
Laboratory of Molecular Genetics, Department of Molecular Biology, Seoul National University, Seoul 151-742, Korea

대장균에서 한국형 B형 간염바이러스 내면항원 유전자의 발현

최수근 · 이원상 · 김성기 · 노현모*
서울대학교 분자생물학과 분자유전학 연구실

ABSTRACT: We cloned and expressed hepatitis B viral core antigen (HBcAg) gene in *E. coli* using P_L promoter system. For optimal expression of the gene, we undertook the studies on the effects of the distance between Shine-Dalgarno (SD) sequence and start codon, copy number of repressor gene, induction temperature, and the stability of the core antigen. The results demonstrated that the induction at 37℃ was more efficient than at 42℃, and the 11 base pairs (bp) distance between SD sequence and start codon of HBcAg gene was more efficient than the 15 bp distance in *E. coli*. The copy number of cI857 repressor gene did not influence on the expression of HBcAg, and the expression level of HBcAg in mutant type (low protease activity) and wild type strains was almost the same. The produced core antigen appeared to be HBcAg not HBeAg judged by two different radioimmunoassay (RIA) kits. This result suggested that the antigen was stable in *E. coli*.

KEY WORDS □ HBV core gene, expression, SD sequence, *E. coli*.

Recent progress in understanding the molecular biology of hepatitis B virus (HBV) has been largely accomplished through the expression of cloned viral DNA in a variety of host cells. The sequence analysis of cloned HBV DNA has resulted in the identification of at least four open reading frames (ORFs); S/pre-S, C, X and P (Robinson *et al.*, 1981).

The C ORF contains two in-phase ATG codons, delimiting the pre-C sequence and the C region. The core protein (p22) assembles with pregenomic viral RNA into a core particle (Will *et al.*, 1987). This particle contains protein kinase activity capable of phosphorylating the p22 protein (Albin and Robinson, 1980; Petit and Pillot, 1985). The N-terminus of p22 contains highly conserved protease-like segment (Asp-Thr-Gly) that is usually present at the gag-pol overlapping region in most hepadnaviruses and retroviruses (Miller, 1987). The C-terminus of p22 is remarkable in that it is extremely rich in arginine, serine and proline residues. A small soluble e antigen (HBeAg) is also encoded by the C gene and produced by the proteolytic cleavage of HBcAg. The HBeAg is secreted out of the cell and the secretion is mediated by the signal sequence of pre-C region. Although many studies were performed to know the function of HBcAg, the function of protein kinase activity and the protease domain of core protein are unknown.

Mutant HBV (*adr*) genome from a Korean patient was cloned previously (Choi *et al.*, 1984) and its whole nucleotide sequence was determined (Rho *et al.*, 1989). The sequence data revealed that a single T addition occurred at the nucleotide 1, 821. This mutation caused the frameshift of X ORF and the precore region. Thus, the virus life cycle may require another mechanism in addition to that of wild-type virus.

It was reported that the differential utilization of codons for particular amino acids between *E. coli* and certain eukaryotes (Grantham *et al.*, 1980) might acount for the observations some eukaryotic cDNAs were expressed at lower levels than expected in *E. coli*. Because the HBcAg gene contains 26 arginine codons, 17 of which are rarely

*To whom correspondence should be addressed.

utilized in *E. coli* (Valenzuela *et al.*, 1980), the overexpression of HBcAg was difficult in *E. coli*. In this study, we investigated the optimum conditions which affect the expression of HBcAg to overcome codon usage.

For the optimal expression of HBcAg, the recombinant plasmids with the appropriate distance between SD sequence and ATG codon of HBcAg gene were constructed. Heat induction was performed at 37℃ or 42℃. We also investigated the stability of HBcAg and the effect of copy number of temperature-sensitive mutant of cI repressor gene (cI857) on the expression of HBcAg. The results indicated that the induction at 37℃ was more efficient for the expression of HBcAg than at 42℃, and that the copy number of cI857 gene did not influence on the expression of HBcAg in *E. coli*. In addition, the HBcAg appeared to be stable and not cleaved to HBeAg in *E. coli*, the size of which was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and radioimmunoassay (RIA).

MATERIALS AND METHODS

Enzymes and reagents

Restriction enzymes were purchased from Bethesda Research Laboratories (BRL), New England Biolabs (NEB) and KOSCO. Other enzymes including mung bean nuclease and T4 DNA ligase were purchased from NEB. Radioimmunoassay (RIA) kits for the detection of HBcAg were purchased from Abbott Laboratories and Sorin Biomedica.

Bacterial strains

E. coli strain M5248 (λ bio275, cI857, H1) was used for the cloning and expression of core antigen gene. The plasmid containing mutant cI gene was transformed into strains HB101 (F⁻, r⁻, m⁻, recA 13), CAG630 (lacZam, trpam, phoam, SupCts, Str⁻, malam, htpR⁻, Tn10 (tet^r)) and LC137 (lacam, trpam, phoam, malam, SupCts, tsx:Tn10). These transformed strains were used as host for the expression of core antigen.

Cell culture and induction

E. coli strains were grown at 30℃ in LB medium and transformed by the method of Kushner (1981). The induction medium for the antigen expression contained 32 g of tryptone and 20 g of yeast extract per liter, with additions of M9 salts (Miller, 1972) and ampicillin to 100 μg/ml. The induction was performed for 2 hr at 42℃, or for 17 hr at 37℃.

Assays of core antigen

After heat induction of the cells harboring the corresponding plasmids, 1.0 ml of the induced cultures were harvested by centrifugation. The cells were resuspended in 500 μl of a sample buffer (1.5% Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 2% 2-mercaptoethanol, 0.01% bromophenol blue), followed by boiling for 5 min. The samples were centrifuged and analyzed by SDS-PAGE. On the other hand, the induced cells were harvested and resuspended in 500 μl of a sonication buffer (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM 2-mercaptoethanol). After freezing and thawing, the 60 μl of samples were assayed with RIA kits from Abbott Laboratories and Sorin Biomedica.

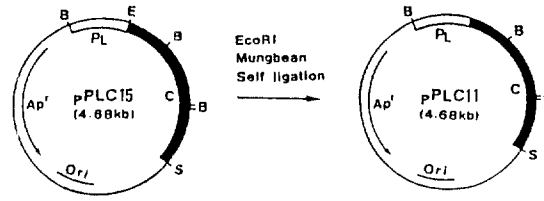

Fig. 1. *Construction of plasmids for expression of HBcAg.*
Each plasmid pPLC15 and pPLC11 has a 15 and 11 base pairs of the distance between SD sequence and start codon of HBcAg gene, respectively. P_L, lambda P_L promoter; C, core antigen gene; E, EcoRI; S, SalI; B, BglII.

RESULTS AND DISCUSSION

Construction of expression plasmids

Since the distance between Shine-Dalgarno (SD) sequence and start codon of structural gene is very important for the expression of foreign gene in *E. coli* (Shine and Dalgarno, 1974), two plasmids, pPLC15 and pPLC11, were constructed with an appropriate distance between SD sequence and start codon of HBcAg gene (Fig. 1). The previously constructed plasmid pUCP1 that has a precore region and core gene was used for the construction of two plasmids pPLC15 and pPLC11 (Choi, 1990; Kim, 1988). The DNA fragment containing precore region and C gene was electroeluted from the plasmid pUCP1 and digested with NlaIII followed by cloning under the control of the P_L promoter. The resulting plasmid, named pPLC15, had a 15 base pairs (bp) of the distance between SD sequence and start codon of HBcAg gene. For the shorter distance, plasmid pPLC15 was digested with EcoRI and sticky ends were removed with the mung bean nuclease digestion followed by self ligation. The restriction analysis showed that the resulting plasmid pPLC11 had a 11bp of the distance between SD sequence and start codon of HBcAg gene.

Expression of HBcAg in *E. coli*

For the expression of core antigen, the recombinant plasmids, pPLC15 and pPLC11, were transformed into the *E. coli* cells. After growth and heat induction, each cell lysate was analyzed by SDS-PAGE and assayed with RIA kits from

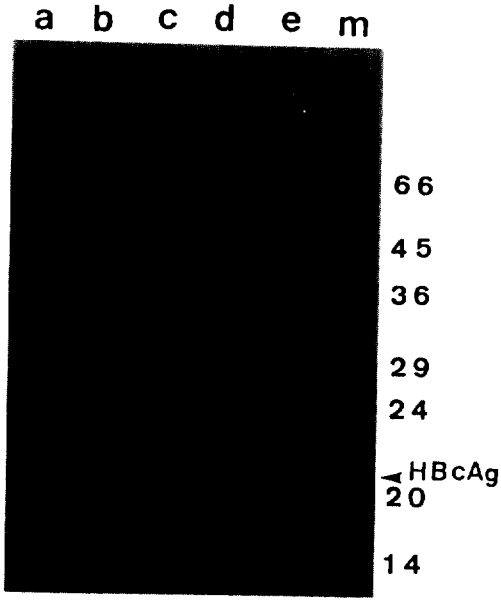

Fig. 2. *Expression of HBcAg in E. coli.*
The plasmids pPLC15 and pPLC11 were transformed into *E. coli* HB101 containing plasmid pRK248. The cell lysates were analyzed by SDS-polyacrylamide gel (12%) electrophoresis and stained by Coomassie Brilliant Blue. The arrow indicates the expressed HBcAg. Each lanes was the temperature-induction products; a, *E. coli* host; b, at 42℃ from pPLC15 transformed cells; c, at 37℃ from pPLC15 transformed cells; d, at 42℃ from pPLC11 transformed cells; e, 37℃ from pPLC11 transformed cells; m, molecular weight marker.

Abbott and Sorin.

Phage lambda P_L promoter is inducible by heat induction commonly performed at 42℃ (Mott *et al.*, 1985; Reinikainen *et al.*, 1988). However, it was reported that the plasmid stability was extremely decreased for long-time induction at 42℃ (Han, 1987). In this reason, the induction was generally performed for short time. In this study, overnight induction at 37℃ was compared with induction for 2 hr at 42℃. The SDS-PAGE (Fig. 2) and RIA results (Table 1) showed that the HBcAg was highly expressed on the overnight induction at 37℃ in comparison with induction for 2 hr at 42℃. This results revealed that long-time induction at 37℃ was more efficient for the expression of HBcAg than the short-time induction at 42℃ in *E. coli*.

HBeAg has been characterized in serum as a 15.5 KD polypeptide, which has been shown by carboxyl-terminal sequence analysis to lack the last 34-36 amino acid residues of core protein (Standring *et al.*, 1988). In this study, we investigated the synthesis of HBeAg by the degradation or proteolytic cleavage of core protein in *E. coli*. The RIA kit from Sorin can not detect the natural HBcAg (Gallina *et al.*, 1989) but detect HBeAg and the RIA kit from Abbott can detect both the HBcAg and HBeAg. The RIA results showed that the expressed antigens were not detected by the Sorin RIA kit but detected by the Abbott RIA kit (Table 1). This result revealed that the core antigens were not cleaved to e antigens in *E. coli*.

The *E. coli* M5248 contains a single copy of the cI857 repressor gene which is integrated into the host genome. However, the cI857 gene, contained

Table 1. *Effect of induction temperature on the expression and stability of HBcAg in E. coli*

Induction temprature[a]	Reactivity with anti-e antibody (cpm)[b]	
	Abbott RIA kit[c]	Sorin RIA kit[d]
control	477	658
37℃	352,280	528
42℃	6,948	377

[a] M5248 carrying the plasmid pPLC15 was induced in maximal induction media for 2 hr at 42℃ and overnight at 37℃, collected, lysed with freezing and thawing, and cleared by centrifugation
[b] Assayed with 60 μl of cell extract (2.5×10^7 cells).
[c] Abbott RIA kit can detect both the HBcAg and HBeAg.
[d] Sorin RIA kit can not detect the natural HBcAg.

Table 2. *Expression of HBcAg in various E. coli strains*

Host[a]	Reactivity with anti-e antibody (cpm)[b]		
	control	pPLC15[c]	pPLC11[c]
M5248	477	352,280	3,455,246
HB101(cIts)	444	339,652	3,435,928
CAG630(cIts)	409	381,250	3,488,902
LC137(cIts)	399	316,794	3,434,712

The cpm of negative control was 374±5 ($n=3$). n is the number of independent measurments. Cutoff value=2.1×374 cpm=785 cpm. Samples with cpm less than the cutoff value are considered nonreactive for HBcAg by the criteria of the Abbott-HBe test.
[a] Strains HB101, CAG630 and LC137 were transformed with the plasmid containing a temperature-sensitive mutant cI gene.
[b] Assayed by AUSRIA-II RIA kit (Abbott) with 60 μl of cell extract (2.5×10^7 cells).
[c] The distance between SD sequence and start codon of HBcAg gene was 15 bp and 11 bp in pPLC15 and pPLC11, respectively.

in a plasmid, would be present in multicopy. To study the effect of copy number of cI857 gene on the expression of HBcAg, we expressed core antigen gene in M5248 which has a single copy of cI857 gene on the host chromosome or HB101 which has plasmid pRK248 containing cI857 gene. The expression level of HBcAg was about the same in these two strains (Table 2). This result indicated that the copy number of cI857 gene did not influence on the expression of core antigen in *E. coli*.

For the study of stability of the HBcAg, we also expressed core antigen gene in CAG630 ($htpR^-$) and LC137 ($htpR^-$, lon^-) cells that have low activity of protease La (Table 2). The expression level of HBcAg in HB101, CAG630 and LC137 was also the same. This result suggests that the HBcAg appears to be stable in *E. coli*.

Since the distance between SD region and start codon affects the translational efficiency (Jay *et al.*, 1982; Shepard *et al.*, 1982), the two plasmids containing the HBcAg gene were constructed with the different distance between SD region and ATG start codon. The cells harboring the plasmid pPLC 11 were expressed higher than the cells containing the plasmid pPLC15 (Table 2). This result agreed to SDS-PAGE data (Fig. 2). Thus, the 11 bp distance betwen SD region and start codon of HBcAg gene was more efficient for the expression of HBcAg than the 15 bp in *E. coli*. This result agrees to the previous observation that the optimal distance between the SD region and the ATG start codon was approximately 11 nucleotide (Dalboge *et al.*, 1988).

Previous evidence showed that the particulated form of core protein had a protein kinase activity, but there is no evidence that the free polypeptide of HBcAg has a protein kinase activity. Also, further studies are required about the function of protein kinase activity on the virus life cycle. For the study of the function of HBcAg, we have undertaken the purification and characterization of expressed HBcAg.

적 요

P_L promoter를 이용하여 대장균 세포내에서 한국형 B형 간염 바이러스의 내면항원 유전자를 클로닝하고 발현시켰다. 유전자의 발현조건을 최적화하기 위하여 P_L 작동유전자의 Shine-Dalgarno (SD) 염기서열과 내면항원의 ATG 사이의 거리, P_L 작동유전자의 억제 유전자의 복제수, 발현유도 온도, 그리고 내면항원의 안정성의 효과에 관하여 연구하였다. 그 결과, 내면항원은 42℃보다는 37℃에서 더 잘 발현되었으며 SD 염기서열과 내면항원의 ATG 사이의 거리가 11 염기 떨어진 것이 15 염기 떨어진 것보다 발현정도가 훨씬 더 높았다. P_L 작동유전자의 억제유전자의 복제수는 내면항원의 발현에 영향을 미치지 않았으며, 단백질 분해효소의 발현이 저해된 숙주세포와 정상적인 숙주세포에서 내면항원의 발현이 거의 같았다. 발현된 내면항원은 두 가지의 방사면역측정법으로 판단한 결과 e 항원이 아니라 C항원인 것으로 나타났다.

사 사

이 논문은 교육부 학술연구조성비 (1990)에 의하여 수행되었다.

REFERENCES

1. **Albin, C. and W.S. Robinson,** 1980. Protein kinase activity in hepatitis B virus. *J. Virol,* **34**, 297-302.
2. **Choi, M.Y., Y.S. Kim, and H.M. Rho,** 1984. Cloning and genetic mapping of the hepatitis B viral genome. *J. Kor. Soc. Virol.,* **14**, 49-55.
3. **Choi, S.K.,** 1990. Expression of hepatitis B viral core and e antigen gene in *Escherichia coli.* M.S. Thesis, Seoul National University.
4. **Dalboge, H., S. Carlsen, E.B. Jensen, T. Christensen, and H.-H.M. Dahl,** 1988. Expression of recombinant growth hormone in *Escherichia coli*: Effect of the region between the Shine-Dalgarno sequence and the ATG initiation codon. *DNA,* **7**, 399-405.
5. **Gallina, A., F. Bonelli, L. Zentilin, G. Rindi, M. Muttini, and G. Milanesi,** 1989. A recombinant hepatitis B core antigen polypeptide with the protamine-like domain deleted self-assembles into capsid particles but fails to bind nucleic acids. *J. Virol.,* **63**, 4645-4652.
6. **Grantham, R., C. Gautier, M. Gouy, R. Mercier, and A. Pave,** 1980. Codon catalog usage and the genome hypothesis. *Nucleic Acids Res.,* **8**, 249-262.
7. **Han, M.H.,** 1987. In Annual Report Submitted to the Ministry of Science and Technology, Korea.
8. **Jay, E., A.K. Seth, J. Rommens, A. Sood, and G. Jay,** 1982. Gene expression: Chemical synthesis of *E. coli* ribosome binding sites and their use in directing the expression of mammalian proteins in bacteria. *Nucleic Acids Res.,* **10**, 6319-6329.
9. **Kim, S.K.,** 1988. Expression and regulation of hepatitis B viral core antigen gene in *Escherichia coli.* M.S. Thesis, Seoul National University.
10. **Kushner, S.R.,** 1981. An improved method for transformation of *E. coli* with colEI-derived plasmids. p. 17. In Genetic Engineering. Elsevier /North-Holland, Amsterdam.
11. **Miller, J.H.,** 1971. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
12. **Miller, R.H.,** 1987. Proteolytic self-cleavage of

hepatitis B virus core protein may generate serum e antigen. *Science*, **236**, 722-725.

13. **Mott, J.E., R.A. Grant, and T. Platt,** 1985. Maxaimizing gene expression from plasmid vectors containing the P_L promoter: Strategied for overproducing transcription termination factor. *Proc. Natl. Acad. Sci.* USA, **82**, 88-92.
14. **Petit, M.A. and J. Pillot,** 1985. HBc and HBe antigenicity and DNA binding activity of major core protein p22 in HBV core particles isolated from the cytoplasm of human liver cells. *J. Virol.*, **53**, 543-551.
15. **Reinikainen, P., M. Lahde, M. Karp, I. Suominen, P. Markkanen, and P. Mantsala,** 1988. Phage lambda P_L promoter controlled α-amylase expression in *Escherichia coli* during fermentation. *Biotechnol. Lett.*, **10**, 149-154.
16. **Rho, H.M., K.T. Kim, S.W. Hyune, and Y.S. Kim,** 1989. The nucleotide sequence and reading frames of a mutant hepatitis B virus subtype *adr*. *Nucleic Acids Res.*, **17**, 2124.
17. **Robinson, W.S., P. Marion, M. Feitelson, and A. Siddiqui,** 1981. The hepa-DNA-virus group: hepatitis B and related viruses, p. 57-68. In Viral Hepatitis (eds. Szzmuness, J. A. and J. Maynard) Franklin Institute Press, Philadelphia.
18. **Shepard, H.M., E. Yelverton, and D. Goeddel,** 1982. Increased synthesis in *E. coli* of fibroblast and leukocyte interferons through alterations in ribosome binding sites. *DNA*, **1**, 125-131.
19. **Shine, J. and L. Dalgarno,** 1974. The 3'-terminal sequence of *E. coli* 16S RNA: Complementarity to nonsense triplets and ribosome binding sites. *Proc. Natl. Acad. Sci. USA*, **71**, 1341-1346.
20. **Standring, D.N., J.H. Ou, F.R. Masiarz, and W.J. Rutter,** 1988. A signal peptide encoded within the precore region of hepatitis B virus directs the secretion of a heterogenous population of e antigens in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA*, **85**, 8405-8409.
21. **Valenzuela, P., Quiroga, M. Zaldivar, J. Gray, P. and Rutter, W.J.,** 1980. The nucleotide sequence of the HBV genome and the identification of the major viral genes. In Animal Virus Genetics (eds. Fields, B.N., R. Jaenisch and L. Fox) pp. 57-71 (Academic Press, New York).
22. **Will, H., W. Reiser, T. Weimer, E. Pfaff, M. Buscher, R. Sprengel, R. Cattaneo, and H. Schaller,** 1987. Replication strategy of human hepatitis B virus. *J. Virol.*, **61**, 904-911.

(Received April 24, 1991)
(Accepted May 9, 1991)