CLONING AND RESTRICTION ENDONUCLEASE ANALYSIS OF THE HEPATITIS B VIRAL GENOME

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ABSTRACT

The HBV-DNA isolated from Dane particles of three cases of hepatitis carriers was successfully cloned into the BamHI site of the pBR322 plasmids. Three recombinant plasmids with full length HBV-DNA from these three individuals were obtained. The comparison between our cloned HBV-DNAs and four clones reported by others revealed the significant restriction sites of endonucleases in our clones. These restriction sites of endonucleases were shown to be useful in the identification of adr *and adw subtypes. All of our clones were also confirmed as adr subtype by the restriction patterns. Moreover, we could find the heterogeneity occurs in the same subtype. However, our clones are very similar to each other in restriction patterns. These results suggested that all of our cloned HBV-DNA might be in the same genotype.*

INTRODUCTION

The widespread infection of hepatitis B virus (HBV) in human being, associated with a variety of liver diseases, is a major worldwide public health problem. It has been estimated that about 200 million people are infected worldwide. However, the prevalence is much higher in some areas of Southeast Asia, the Middle East and Africa[1]. In Thailand, HBV is also one of the public health problems with the infection rate about 40-50 percent and about 8-10% of the population are hepatitis carriers.

Recent studies have revealed that HBV-DNA is the smallest double-stranded DNA viral genome of known mammalian viruses. Its genome is comprising of a long strand of about 3,200 nucleotides and a short strand region encompassing 15 to 50% of the length of the whole genome[2]. The endogenous viral DNA polymerase reaction is responsible for the conversion of this short stranded region to a complete double-stranded DNA. There are several particulate forms of HBV found in the sera of acute and chronic hepatitis patients. They are consisting of the 42 nm Dane particles, the 22 nm sphere and filaments of hepatitis B surface antigen (HBsAg)[3]. The Dane particle is known as an infectious and complete virion. It is composing of two layers of antigens. The outer coat envelope is known as HBsAg while the inner coat is the nucleocapsid containing HBV-DNA inside. The inner coat is also known as hepatitis core antigen (HBcAg). For HBsAg, it is serological classified into four major subtypes, *adw*, *adr*, *ayw* and *ayr*. Interestingly, the distribution of subtypes shows geographic characteristics[4]. In Thailand, we found only *adr* and *adw* subtype in the proportion of 81.0% and 19.0% respectively[5].

There are several limiting factors in studying the hepatitis B virus. The first factor is the inability of HBV virus to replicate in established cell lines or standard laboratory animals. The another factor is the limited amount of HBV-DNA available for the study. However, the propagation and detailed analysis of the structure of the viral DNA can be manipulated by gene cloning and sequencing techniques. Although several subtypes of HBV had been cloned and sequenced[6-10], our goal was to clone and analyze the HBV-DNA of major HBV subtype found in Thailand. The study of the expression of HBV gene products in either prokaryotic or eukaryotic system is the ultimate goal of this study by using these clones as a source of material. The other alternatives are for the production of HBV-DNA probe and viral antigens for diagnostic purpose, and possibly for vaccine development. Our findings suggested that the subtype of HBV (*adw*, *adr*, *ayr* and *ayw*) could be distinguished from each other by using restriction endonucleases. The cloned HBV-DNAs in our laboratory were *adr* subtypes and its heterogeneity could be found in the same subtype.

MATERIALS AND METHODS

1) Cloning of full length HBV-DNA into plasmid for E. coli.

1.1) Full length HBV-DNA.

The complete virions of HBV that were purified from blood samples obtained from the blood donors were used. The incomplete double stranded DNA in the nucleocapsid of the complete virion was allowed to repair by the endogenous DNA polymerase reaction[11]. The repaired mechanism of endogenous DNA polymerase was done in the presence of four deoxyribonucleotide triphosphates. The purified HBV-DNA obtained from repaired mechanism by endogenous DNA polymerase reaction was used for cloning into the plasmid.

1.2) Plasmid.

The plasmid vector used for cloning the whole genome of HBV-DNA was pBR322. This plasmid is 4.36 kb in size and contains resistant genes to tetracycline (tet^R) and ampicillin (amp^R) and restriction sites of endonucleases. The genetic map of the plasmid pBR322 is shown in figure 1.

1.3) Construction of the recombinant plasmid.

In the study, whole complete double-stranded viral genome that was previously identified as *adr* subtype by polymerase chain reaction[5] was used for gene cloning. It was reported that there was one restriction site for BamHI endonuclease located at nucleotide position 1,274 in HBV-DNA of *adr* subtype where the *XhoI* site is the start position 1[12]. The *Bam*HI site of the pBR322 plasmid is located at nucleotide 375 as shown in Figure 1. Therefore, the whole complete double-stranded viral genome and pBR322 were digested with *Bam*HI endonuclease to make them linear forms. The linear pBR322 plasmid was then dephosphorylated by calf intestinal alkaline phosphatase (CIP). The dephosphorylated pBR322 and digested HBV-DNAs were ligated with T4 DNA ligase to yield recombinant pBR322 plasmid (*r*-pBR322). This constructed recombinant plasmid was expected to carry the whole genome of HBV-DNA.

1.4) Transformation.

The constructed r-pBR322 was transformed into *E. coli* strain DH5α according the method described by Hanahan[13]. A culture of *E. coli* was grown until it reached mid log phase where the OD 600 value was 0.6. Then, a 50 ml culture of *E. coli* was centrifuged for 10 minutes at

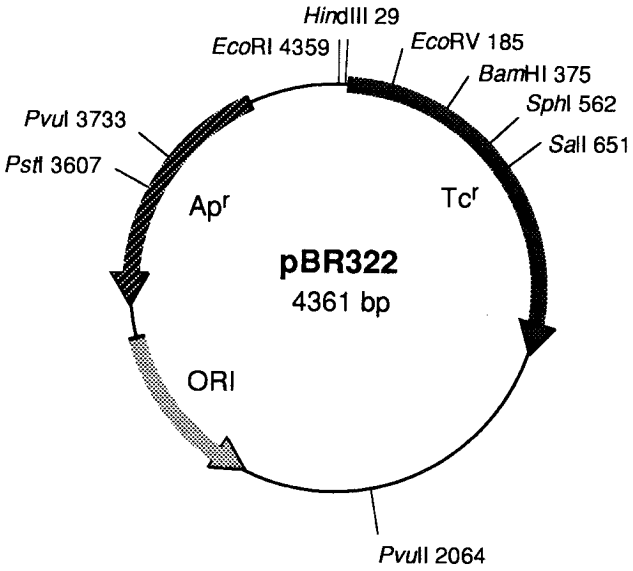

Fig. 1. Schematic diagram of genetic map of pBR 322 showing tet^{γ} gene, amp^{γ} gene and restriction sites of endonucleases.

4°C. The SOB medium containing 20 mM $MgSO_4$ was used as the growth medium of *E. coli*. The cell pellet was re-suspended in 18 ml of cold FSB buffer and kept at 0°C for 15 minutes. The FSB buffer was consisting of 10 mM Potassium acetate, 45 mM $MnCl_2$. 4H2O, 10 mM $CaCl_2$. $2H_2O$, 100 mM KCl, 3 mM Hexamminecobalt chloride and 10% glycerol. These competent cells were harvested by centrifugation and re-suspended in 2 ml of FSB buffer and incubated on ice for 2 hours. A 50 nanograms aliquot of *r*-pBR322 plasmids was added to 0.2 ml of the competent cells and the mixture was left on ice for 30 minutes. One ml of SOC medium was added to a mixture when the mixture had been treated at 42°C for two minutes and the mixture was subsequently incubated at 37°C for one hour. The transformants were screened by growing on SOB agar plates containing 50 μg/ml of ampicillin incubated at 37°C for 12-16 hours. Individual colonies were selected for replica plating by growing in both SOB agars containing 50 μg/ml of ampicillin and SOB agar containing 40 μg/ml of tetracycline. The colonies that did not grow in the presence of tetracycline but did grow on the plate containing ampicillin should contain *r*-pBR322 plasmids because the tet^R gene had been inactivated. These *r*-pBR322 plasmids were most likely to carry full-length genome of HBV. These colonies of transformants were propagated and kept for further characterization.

2) Characterization of the transformants.

In this study, three transformants were obtained by using HBV-DNAs derived from different individual blood samples. These 3 transformants were designated as pDKC1, pDKC2 and pDKC3, respectively. Their plasmids were characterized for the insertion of the full length of HBV-DNAs as follows. They were subsequently propagated and their plasmids were purified. These plasmids were then digested with BamHI endonuclease and the restriction analysis was studied. Their DNA bands were detected by using gel electrophoresis with ethidium bromide staining and southern blot hybridization with HBV-DNA probes labeling with chemiluminescence (ECL). The detection was done under detection system of Amersham, UK.

3) The heterogeneity of HBV-DNA.

The restriction endonuclease analysis was used to study the heterogeneity of HBV-DNAs derived from pDKC1, pDKC2 and pDKC3 in comparison with those clones that had been reported by others. Ten restriction endonucleases; *Bam*HI, *Bgl*II, *Hpa*I, *Kpn*I, *Xho*I, *Pst*I, *Hin*dIII, *Eco*RI, *Xba*I and *Hae*II were used in the study. The results of restriction patterns of HBV-DNAs digested with ten endonucleases were observed by gel electrophoresis and southern blot hybridization. All the restriction sites of endonucleases in our clones were also summarized and compared with other reported clones. Four clones of reported subtypes were used. They were consisting of 2 clones of *adr* subtype and 2 clones of *adw* subtype. The two clones of *adr* subtype(S1 and S2) were those reported by Ono *et al.*[7] and Fujiyama *et al.*[14] respectively. The other two clones of *adw* subtype(S3 and S4) were those reported by Valenzuela *et al.*,[9] and Ono *et al.*[7], respectively.

RESULTS

The full length of complete double stranded HBV-DNA of *adr* subtype derived from three different blood samples were successfully cloned into *E. coli* strain DH5α. The entire HBV-DNA molecule of viral genome was cut with *Bam*HI endonuclease and ligated at the *Bam*HI site located in the tet^R gene of the pBR322 plasmid. The transformants with amp^R gene were selected and screened for the sensitivity to tetracycline. It was found that about 20 % of the ampicillin resistant clones were sensitive to tetracycline. This result indicated that there was the insertion of the foreign gene in the tet^R gene.

Three clones were selected and designated as pDKC1, pDKC2 and pDKC3, respectively. These selected clones were then isolated and theirs plasmid DNAs were examined by agarose gel electrophoresis after *Bam*HI cleavage. Results are shown in figure 2. It was found that these selected three clones showed two bands of linear DNAs at 4.3 kb of pBR322 plasmid and 3.2 kb of HBV-DNA after BamHI digestion (lane B). However, only one band of HBV-DNA was observed at 3.2 kb after hybridization with HBV-DNA probes. This result indicated that the entire HBV genome was inserted in the plasmid of all individuals of pDKC1, pDKC2 and pDKC3 clone.

To study the heterogeneity of these three clones, their plasmid-DNAs were examined by restriction endonuclease analysis. The restriction patterns of the pDKC1 clone after digestion with ten endonucleases were summarized in Figure 3. The patterns were consisting of 7.5 kb of linear DNA after *Bgl*II, *Hpa*I, *Kpn*I, *Xho*I, *Pst*I and *Hin*dIII cleavages. This meant that the HBV-DNA insert had only one restriction site for *Bgl*II, *Hpa*I, *Kpn*I and *Xho*I whereas there was no restriction site for *Pst*I and *Hin*dIII endonucleases. These results agreed with pBR322 plasmid that had no restriction site for the above four endonucleases except one restriction site for *Pst*I and *Hin*dIII endonucleases (see Figure 1). However, the patterns of the clone pDKC1 showed two DNA-bands when digested with either *Bam*HI or *EcoRI* or *Xba*I endonucleases. The sizes of DNA were 4.3 and 3.2 kb after *Bam*HI cleavage; 6.0 and 1.5 kb after *EcoRI* cleavage; and 6.5 and 1.0 kb after *Xba*I cleavage. The findings suggest that the HBV-DNA has only one restriction site for *Bam*HI and *EcoRI* whereas there are two restriction sites of *Xba*I endonuclease. This is based on the fact that there is one restriction site for *Bam*HI and *EcoRI* but there is no restriction site for *Xba*I endonuclease locating in pBR322. Moreover, many restriction patterns of clone pDKC1 was observed after *Hae*II digestion. Since, it appeared only two DNA-bands of HBV-DNA at about 1.5 and 1.7 kb after southern blot hybridization. This meant that there was only one restriction site for HaeII endonuclease locating in HBV-DNA. In similar fashion,

Table 1. Summary of restriction sites of ten endonucleases in our 3 selected clones (pDKC 1, pDKC 2 and pDKC 3) and other 4 reported clones (S_1, S_2, S_3, and S_4,) from other countries.

Enzyme	pDKC1	pDKC2	pDKC3	adr (s1)	adr (s2)	adw(s3)	adw(s4)
*Bam*HI	1	1	1	1	1	2	2
*Bgl*II	1	1	1	2	2	3	4
*Hpa*I	1	1	1	0	0	1	1
*Kpn*I	1	1	1	ND	ND	ND	ND
*Xho*I	1	1	1	1	1	0	0
*Pst*I	0	0	0	0	0	1	1
*Hin*dIII	0	0	0	0	0	0	0
EcoRI	1	0	1	1	0	1	1
*Xba*I	2	2	2	1	1	1	1
*Hae*II	1	1	1	2	1	1	1

the restriction patterns of endonucleases of pDKC2 and pDKC3 clones were also studied. The results were summarized and compared with other clones reported from other countries as shown in table 1.

DISCUSSION

In this study, three clones, that is, pDKC1, pDKC2 and pDKC3, were selected as the representative clones that derived from the whole genome of *adr* subtype of different hepatitis carriers. The pBR322 plasmid was used as a plasmid vector to construct the recombinant plasmid and the constructed recombinant plasmid was transformed into *E. coli* strain DH5α. Results of analysis of these clones revealed that they had correct orientation of HBV-DNAs inserted into pBR322 plasmids and had stability in replication of their plasmids. All the clones contained complete double stranded HBV-DNAs with the appearance approximately at 3.2 kb in size after *Bam*HI endonuclease digestion. This finding is similar to that reported by others[7,9].

Our clones had been preliminary identified as *adr* subtype by PCR[5]. However, the 3.2 kb *Bam*HI fragments found in clones pDKC1, pDKC2 and pDKC3 had several interesting features when compared with the restriction maps of clones reported by other laboratories. The restriction site patterns of the cloned HBV-DNAs in comparison with reported cloned from other laboratories are summarized in Table 1. All three clones of *adr* subtype, that is, pDKC1, pDKC2 and pDKC3, two clones(S1 & S2) of *adr* subtype and two clones (S3 & S4) of *adw* subtype were used for comparison. Two features were to be noted. First, we could distinguish the subtype of HBV by restriction mapping. This based on the fact that they appeared to be consistently different between *adr* and *adw* subtype in our comparison. Second, all the *adr* subtypes have one restriction site for both of *Bam*HI and *Xho*I endonucleases whereas there is no restriction site for PstI endonuclease. On the other hand, it has been shown that the *adw* subtype has two restriction sites for *Bam*HI endonuclease, and one restriction site for *Pst*I endonuclease whereas there is no restriction site for *Xho*I endonuclease. Moreover, the

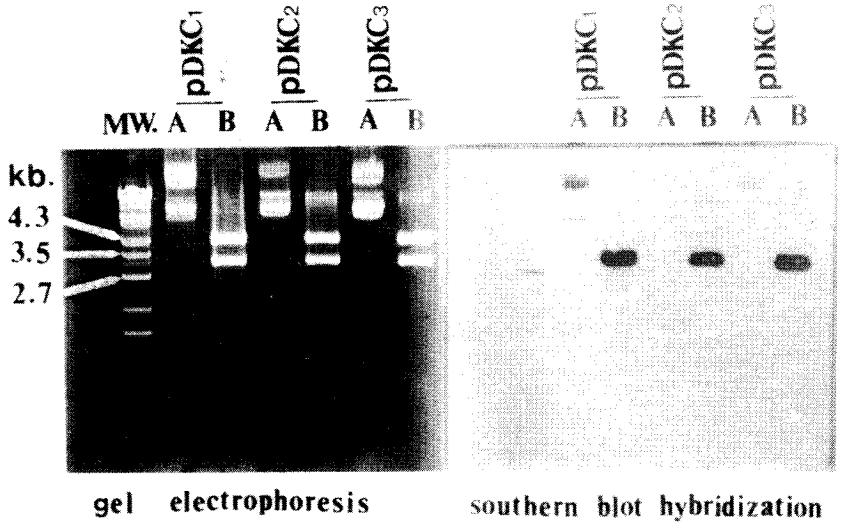

Fig. 2. Agarose gel electrophoresis and southern blot hybridization of DNA patterns of recombinant pBR322 plasmids: clones pDKC1, pDKC2 and pDKC3, digested with *Bam*HI endonuclease

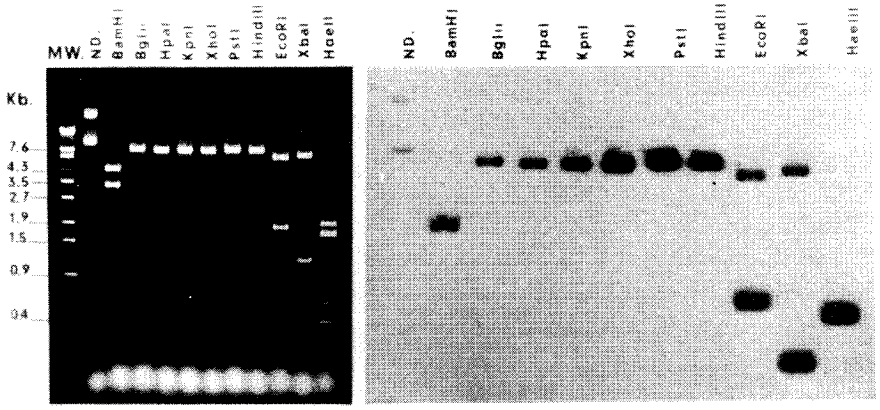

Fig. 3. Agarose gel electrophoresis and southern blot hybridization of restriction patterns of clone pDKC1, digested with ten endonucleases : *Bam*HI, *Bgl*II, *Hpa*I, *Kpn*I, *Xho*I, *Pst*I, *Hin*dIII, *EcoRI*, *Xba*I and *Hae*II.

patterns in *adr* subtype had the heterogeneity among each other. In conclusion, first, it was found that there was one restriction site for both of *Bam*HI and *Xho*I endonucleases and no restriction site for neither *Pst*I nor *Hin*dIII endonucleases in all *adr* subtypes. Second, the heterogeneity of HBV genome in *adr* subtype was observed in some restriction sites, that is, *Bgl*II, *Hpa*I, *Xba*I, *Hae*II and *EcoRI* endonucleases. These differences may be resulting from genomic divergence that suggests to be in different? genotypes. However, our clones are very similar in restriction mapping among each other with only one variation in EcoRI site. Therefore, our clones might be in the same genotype since the same genotype should have gene variation less than 8% of the genome sequence in "s" region of major HBsAg protein[15].

From the above results, the only disadvantage is that the number of available restriction sites of endonucleases is too small to permit a definitive conclusion. However, these results are strongly suggested that simple restriction mapping might be sufficient to identify the subtype of HBV-DNA. Our clones were also confirmed as *adr* subtype by using restriction endonuclease analysis. Moreover, our cloning method allowed the entire HBV-DNA of *adr* subtype to be cloned since there is only one restriction site for *Bam*HI endonuclease at 1274 position on the viral genome that located next to the stop codon of "s" gene. Therefore this method is quite useful and allows us to use these clones for further study in gene sequencing and gene expression of hepatitis B surface antigen (HBsAg) protein.

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บทคัดย่อ

ได้ทำการโคลนยีนของไวรัสตับอักเสบชนิด บี ที่แยกได้จากอนุภาคไวรัส ซึ่งได้มาจากเลือดของคนที่เป็นพาหะของโรคตับอักเสบ บี 3 ราย ใส่เข้าไปในพลาสมิด pBR322 ตรงตำแหน่งที่ถูกตัดด้วยเอนไซม์ *Bam*HI ได้พลาสมิดลูกผสมที่มียีนของไวรัสตับอักเสบบีอยู่ด้วย รวม 3 โคลน เมื่อทำการเปรียบเทียบลักษณะการถูกตัดด้วยเอนไซม์ endonuclease ระหว่างโคลนที่ได้ทั้ง 3 โคลนนี้ กับโคลนที่มีผู้รายงานไว้แล้วจากแหล่งอื่น พบว่าสามารถเลือกใช้ เอนไซม์ endonuclease บางชนิด ในการจำแนกชนิดของไวรัสตับอักเสบชนิดบีที่ได้ ว่าเป็น subtype *adr* หรือ *adw* โดยเปรียบเทียบลักษณะของยีนที่ถูกตัดแล้ว ให้ผลแตกต่างกันและเป็นลักษณะเอกลักษณ์ของแต่ละโคลน นอกจากนี้ ยังพบว่า ในไวรัสตับอักเสบบี ชนิดเดียวกัน ให้ผลแตกต่างกันหลายแห่งเมื่อใช้เอนไซม์ endonuclease หลายชนิดศึกษาเปรียบเทียบกัน อย่างไรก็ตาม พบว่าโคลนที่ได้นั้น มีความจำเพาะต่อการถูกตัดด้วยเอนไซม์ใกล้เคียงกัน ทำให้คิดว่า โคลนที่ได้เหล่านี้น่าจะอยู่ใน genotype เดียวกัน