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The complete nucleotide sequences of the cloned hepatitis B virus DNA; subtype adr and adw

Yoshitaka Ono*, Haruo Onda*, Reiko Sasada*, Koichi Igarashi*, Yukio Sugino* and Kusuya Nishioka+

*Biotechnology Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Jusohonmachi 2-17-85, Yodogawa-ku, Osaka 532 and +Tokyo Metropolitan Institute of Medical Science, Honkomagome 3-18-22, Bunkyo-ku, Tokyo 113, Japan

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ABSTRACT

The complete nucleotide sequences of two different subtypes (adr and adw) of hepatitis B virus (HBV) DNA cloned in E. coli were determined. The sequence of the viral genome of the adr clone was 3188 nucleotides long, and that of the adw clone was 3200 nucleotides long. The adr and adw clones differed from the reported cloned ayw HBV DNA (3182 nucleotides long) in 11.2% and 10.0% of nucleotides, respectively. Heterogeneity of the HBV genome in the clones with the same subtype was observed.

INTRODUCTION

Viral hepatitis, especially that caused by infection with hepatitis B virus (HBV), is a major worldwide public health problem. HBV is a 42 nm particle (Dane particle) consisting of a core containing the viral genome (3200 base pairs (bp) of partially single stranded DNA) bound to the core protein and its own DNA polymerase (1,2); there are at least four subtypes (adw, adr, ayw, and ayr) of HBV that can be recognized by antigenic differences in the surface antigen (HBsAg)(3). However, since the virus has not been propagated in cultured cells, the biological study and preparation of large quantities of HBsAg, which could be used as a vaccine against HBV infection, have been hampered.

To resolve this problem, it is necessary to analyze the viral genome with a defined subtype. Recently the viral genomes were cloned in E. coli (4,5,6), and the nucleotide sequences were determined(7,8,9,10). The complete nucleotide sequence of the subtype ayw HBV DNA has been reported by Galibert et al.(8). Although Valenzeula et al.(10) also reported the complete nucleotide sequence of HBV DNA , they did not use a defined subtype of HBV, but speculated the subtype by congruence of the restriction endonuclease cleavage pattern with that of an adw2 HBV DNA (4). Information on the nucleotide sequence of the other subtypes of HBV is very limited. We prepared HBV DNA from the Dane particles with defined HBsAg subtypes, adr and adw (these subtypes of HBsAg were mainly found in the Southeast Asia and Japan (11,12,13,14)), and cloned each HBV DNA in E. coli. In this paper, we report the nucleotide sequences of the cloned HBV DNA, and discuss the differences in the nucleotide sequences among the different HBV subtypes.

MATERIALS AND METHODS

Enzymes and reagents

All restriction endonucleases were purchased from Takara Shuzo. E. coli DNA polymerase I large fragment and bacterial alkaline phosphatase were obtained from Bethesda Research Laboratories. T4 DNA ligase was purchased from New England Biolabs. The enzymes were used as recommended by the suppliers. All reagents for DNA sequencing were supplied by Bethesda Research Laboratories.

Preparation of HBV DNA

Subtype adr HBV Dane particles were isolated from the plasma (20 ml) of a single donor infected persistently with HBV that was defined as subtype adr of HBsAg, and subtype adw HBV Dane particles were isolated from the plasma(20 ml) of a adw HBV-infected chimpanzee as described previously(15). The single stranded region of HBV DNA was repaired by an endogeneous DNA polymerase reaction according to Kaplan et al.(16). The repaired double stranded viral DNA was extracted using a proteinase/SDS/phenol procedure(17).

Cloning of HBV DNA in E. coli.

HBV DNA (subtype adr), made double stranded and purified from the Dane particles, was digested with BamH1 and ligated to BamH1 digested, alkaline phosphatase treated pBR322 by T4 DNA ligase. Transformation of E. coli χ1776 was carried out according to Enea et al. (18). For cloning of adw HBV DNA, viral DNA was digested with EcoR1, and cloned in the pBR322 EcoR1 site. Recombinant plasmid DNA was prepared from each transformant by the alkaline-SDS procedures (19). The plasmids, which contained approximately 3200 bp inserts, were screened by digestion with BamH1 or EcoR1. One of these plasmids, which was thought to contain the full length of HBV genome, was named pHBr330 as the subtype adr clone, and another was referred to as pHBV933 for the subtype adw HBV clone. pHBr330 and pHBV933 were used as the DNA sources for the determination of the nucleotide sequences.

DNA sequence determination

Plasmid DNA was prepared from each clone by alkaline-SDS procedures followed by equilibrium density gradient centrifugation. In order to determine the nucleotide sequences by the dideoxy chain termination methods (20,21,22), approximately 3200bp inserts of pHBr330 and pHBV933 were digested with several restriction enzymes. The digested fragments were subcloned in M13mp7 phage. Using this M13mp7 phage subclone bank, the cloned HBV DNAs were sequenced. Preparation of the recombinant single stranded phage DNA as a template, DNA polymerase reaction, and polyacrylamide-urea gel electrophoresis were performed according to the manuals of the sequencing kit supplied by Bethesda Research Laboratories. The strategies for sequencing of the pHBr330 and pHBV933 inserts are shown in Fig. 1.

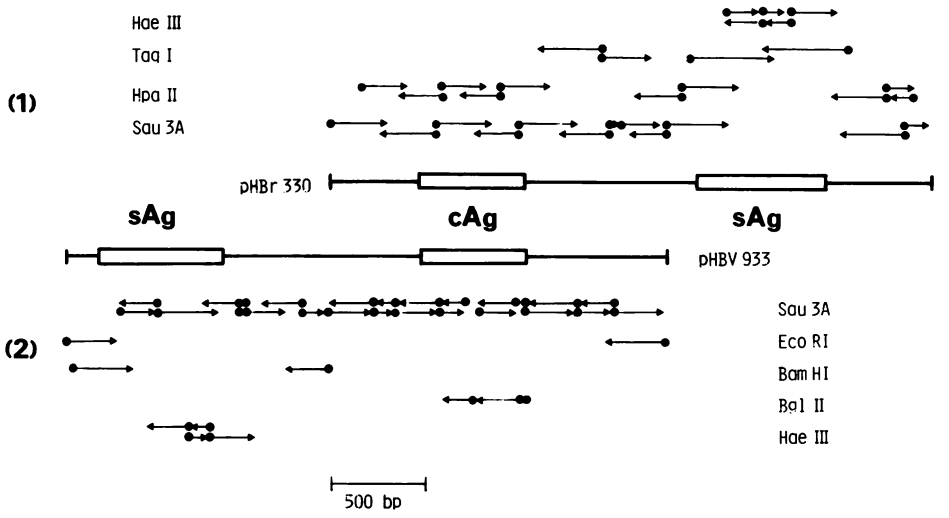


Fig. 1. The sequence strategies for the cloned HBV DNA
(1) adr clone, pHBr330 insert, (2) adw clone, pHBV933 insert.
The open boxes represent the coding regions of HBsAg and HBcAg. The dots indicate the position of each restriction endonuclease cleavage site, and the arrows indicate the directions and ranges of sequence determination. Only restriction endonuclease cleavage sites used for sequence are shown.

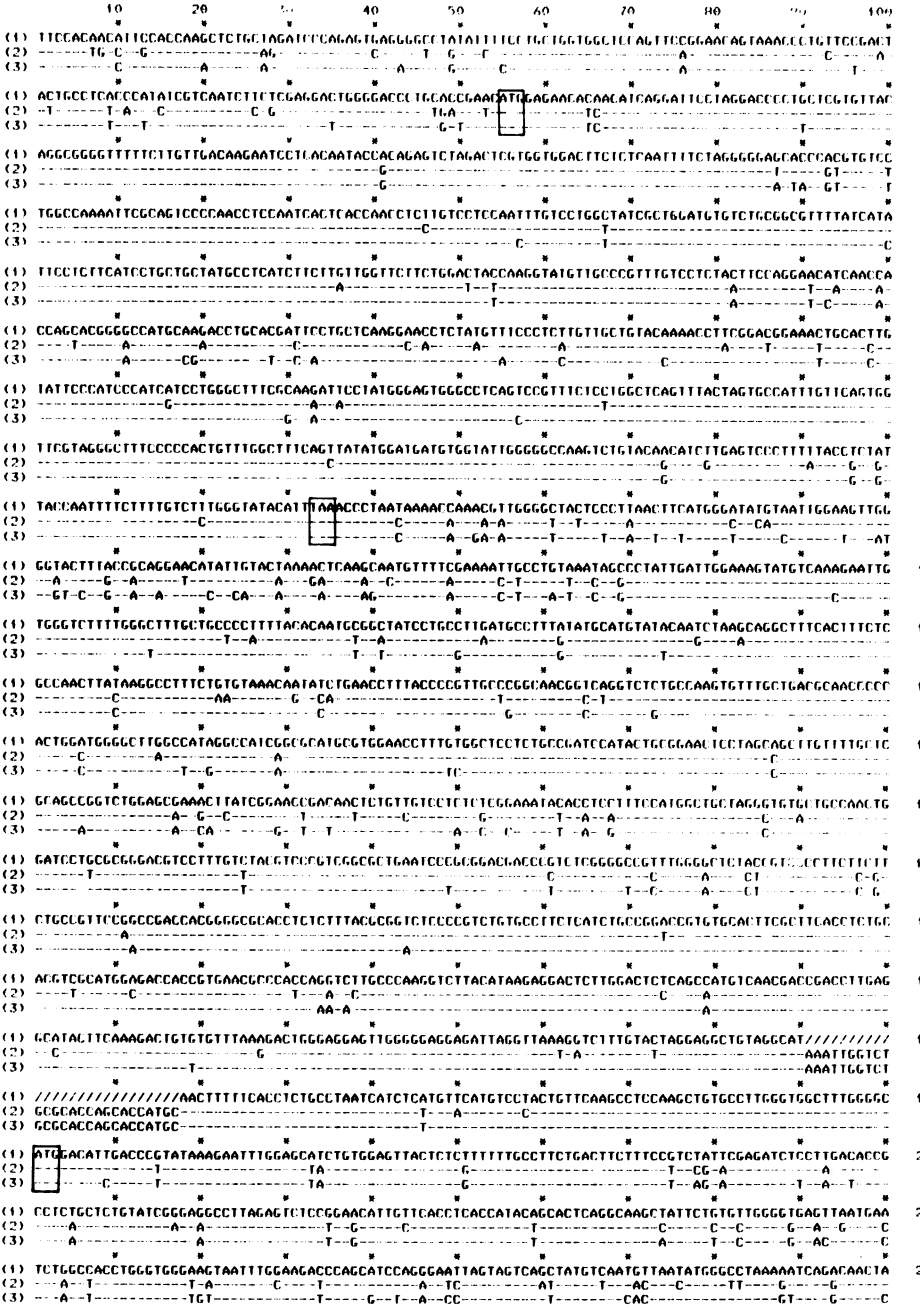
RESULTS

Cloning of subtype adr and subtype adw HBV DNA

In order to clone the full length of HBV DNA, we first of all searched for the restriction enzymes that digested only one position on the viral genome. As reported for the subtype of ayw or adw2 HBV DNA, adw HBV DNA was found to have an unique EcoR1 site (data not shown). So we chose the EcoR1 site of pBR322 for the cloning of adw HBV DNA. On the other hand, the greatest portion of our adr HBV DNA was not digested with EcoR1, but was digested with BamH1 at one position. We, therefore, cloned the subtype adr HBV DNA at the BamH1 site of pBR322 (data not shown). The recombinant plasmids which contained about 3200bp inserts were chosen by the size selection of inserts. From these clones, pHBr330 and pHBV933 were chosen as the DNA source for the DNA sequencing of the subtype adr and adw HBV DNA, respectively.

DNA sequencing analysis

The nucleotide sequences of pHBr330 insert (adr clone) and pHBV933 insert (adw clone) were determined by the dideoxy chain termination procedure employing templates derived from single stranded M13mp7 phage derivatives that were a subclone bank prepared from each insert DNA. As shown in Fig. 2, the sequence of the cloned adr HBV DNA was 3188 nucleotides long, and that of the adw clone was 3200 nucleotides long.



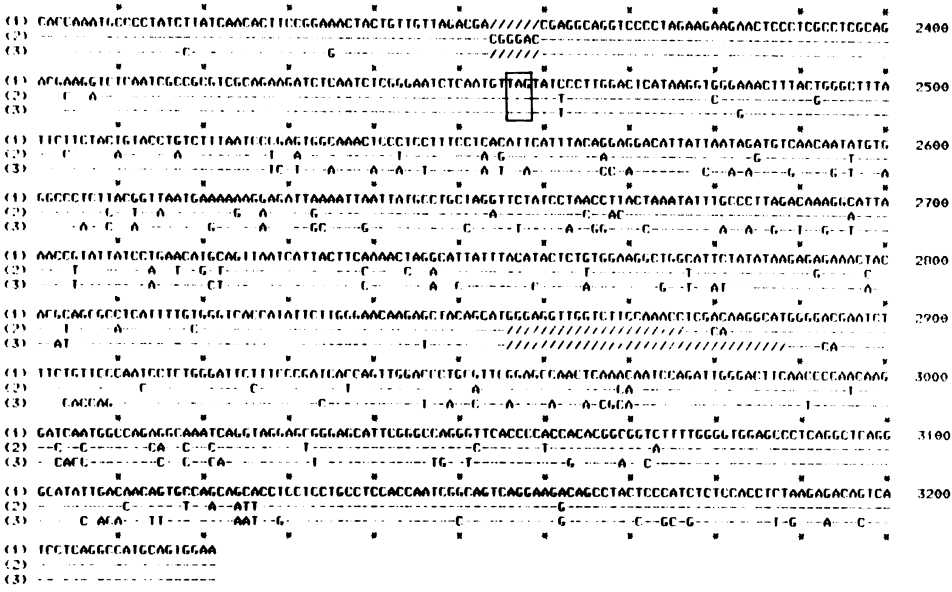


Fig. 2. Comparison of the nucleotide sequences among the different subtypes of HBV DNA. (1) adr clone, pHBr330 insert, (2) adw clone, pHBV933 insert, (3) the reproted ayw HBV DNA(8). In order to compare the three sequences, the sequences are aligned at the EcoR1 site. The nucleotide numbers are tentative, because the deletion and additions exist in each sequence. The bars indicate the same nucleotide as in lane (1). The oblique lines indicate deletions. The initiation codons and the stop codons of HBsAg and HBcAg are boxed.

The adr subtype differed from the reported ayw HBV DNA (3182bp) by deletion of 27bp (1791-1817) and the addition of 33bp (2856-2888), while the adw subtype differed from ayw by the addition of 18bp (2354-2359 and 2877-2888). A two by two analysis of the three nucleotide sequences (adr,adw,and ayw) demonstrated a relatively high degree of divergence. The nucleotide differences were 11.2% between adr and ayw, 10.0% between adw and ayw, and 9.8% between adr and adw. No region contains a marked divergence or a mutational hot spot, except for the deletion and addition of the sequences noted above. Our adw HBV DNA sequence differed from the adw2 sequence reported by Valenzuela et al.(10) by only 1.6% of the nucleotides.

Search for the viral antigen coding regions

Galibert et al.(8), Valenzuela et al.(10), and Pasek et al.(7) have mapped the genes of the two major viral antigens, HBsAg and HBcAg(core antigen), in the HBV genome. We searched for these antigen coding regions in the adw and adr HBV genomes. According to the investigators mentioned above, the HBsAg gene is located between 155 and 832, and consisted of 678 nucleotides coding for 226 amino acids in both HBV subtype

genomes. The amino acid sequences deduced from the nucleotide sequences of the three subtype antigens are shown in Fig. 3. The overall amino acid difference was 7.5% (17 amino acids changes/226 amino acids), but there were relatively large differences in the central hydrophilic region between amino acid residues 113 to 143; 6 changes (19.4%) between adr and adw, 8 changes (25.8%) between adw and ayw, and 7 changes (22.6%) between adr and ayw. Recently, Peterson et al.(23) determined the amino acid sequences of the HBsAg of the adw and ayw subtypes, and demonstrated that amino acid residues 122 to 150 of each antigen occupy an exposed region of the HBsAg lipoprotein particle and the two subtypes were found to differ at two specific position in this region. Therefore, this region might be an antigenically important area of the HBsAg.

HBcAg gene in the adr and adw HBV genome was located at the same region as that in the ayw genome. While the HBcAg gene in the subtype adr HBV genome consisted of 183 amino acids as the subtype ayw, two additional amino acids were observed on the adw HBV genome between residues 151 and 152 (Fig. 4). This insertional sequence was the same as that of adw2 described by Valenzuela et al.(10). The amino acid differences were relatively smaller than those of HBsAg and there was no region of marked divergence (about a 4% difference among the three subtypes).

One of the other viral coded proteins, DNA polymerase, was speculated to be coded in the longest open region, that overlapped the surface antigen gene(8,10). We found a long continuous nucleotide sequence devoid of a stop codon that began at ATG(2307-2309) and ended at a stop codon TGA(1621-1623) in the adr HBV genome. However, in this region of adw HBV genome a stop codon of TAA(550-552) appeared.

Heterogeneity of the cloned HBV genome

When double stranded Dane particle DNA, defined as subtype adr, was digested with EcoR1, almost all the molecules seemed to be undigestable. Nevertheless, on the DNA sequencing data, the pHBr330 insert has the cutting site of EcoR1. This phenomenon might indicate that heterogeneity of the HBV genome existed in the same subtype prepared from a single donor. We, therefore, attempted to characterize other clones obtained from transformants by the chemeric plasmid pHBr series. As shown in Fig. 5, some variation of the restriction patterns was observed in the three recombinant plasmids. Only pHBr330 insert was digested with EcoR1. It is interesting that pHBr329 insert was digested with HindIII, because all of the known HBV DNA was not digested with HindIII (5,24,25).

Although we did not determine the precise nucleotide sequences of pHBr329, HindIII site might be located at 1138-1143. As the sequence of this region of pHBr330 insert was AACCTT, the HindIII site (AAGCTT) could be created by one base substitution. We also observed differences of the restriction patterns in the adw clones (data not shown).

```
(1)  Met Glu Asn Thr Thr Ser Gly Phe Leu Gly Pro Leu Leu Val Leu
(2)   -   -   -  Ile  -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -  Ile  -   -   -   -   -   -   -   -   -   -   -

(1)  Gln Ala Gly Phe Phe Leu Leu Thr Arg Ile Leu Thr Ile Pro Gln       30
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

(1)  Ser Leu Asp Ser Trp Trp Thr Ser Leu Asn Phe Leu Gly Gly Ala
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -  Ser
(3)   -   -   -   -   -   -   -   -   -   -   -   -   -   -  Thr

(1)  Pro Thr Cys Pro Gly Gln Asn Ser Gln Ser Pro Thr Ser Asn His       60
(2)   -  Val   -  Leu   -   -   -   -   -   -   -   -   -   -   -
(3)  Thr Val   -  Leu   -   -   -   -   -   -   -   -   -   -   -

(1)  Ser Pro Thr Ser Cys Pro Pro Ile Cys Pro Gly Tyr Arg Trp Met
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -  Thr  -   -   -   -   -   -   -

(1)  Cys Leu Arg Arg Phe Ile Ile Phe Leu Phe Ile Leu Leu Leu Cys       90
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

(1)  Leu Ile Phe Leu Leu Val Leu Leu Asp Tyr Gln Gly Met Leu Pro
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

(1)  Val Cys Pro Leu Leu Pro Gly Thr Ser Thr Thr Ser Thr Gly Pro      120
(2)   -   -   -   -  Ile  -   -  Ser Thr  -   -   -   -   -   -
(3)   -   -   -   -  Ile  -   -  Ser  -   -   -   -   -   -   -

(1)  Cys Lys Thr Cys Thr Ile Pro Ala Gln Gly Thr Ser Met Phe Pro
(2)   -   -   -   -   - Thr  -   -   -   - Asn  -  Lys  -   -
(3)   - Arg  -   -  Met Thr Thr  -   -   -   -   -   - Tyr  -

(1)  Ser Cys Cys Cys Thr Lys Pro Ser Asp Gly Asn Cys Thr Cys Ile      150
(2)   -   -   -   -   -   -   - Thr  -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

(1)  Pro Ile Pro Ser Ser Trp Ala Phe Ala Arg Phe Leu Trp Glu Trp
(2)   -   -   -   -   -   -   -   -   - Lys Tyr  -   -   -   -
(3)   -   -   -   -   -   -   -   - Gly Lys  -   -   -   -   -

(1)  Ala Ser Val Arg Phe Ser Trp Leu Ser Leu Leu Val Pro Phe Val      180
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   - Ala  -   -   -   -   -   -   -   -   -   -   -   -

(1)  Gln Trp Phe Val Gly Leu Ser Pro Thr Val Trp Leu Ser Val Ile
(2)   -   -   -   -   -   -   -   -   -   -   -   -   - Ala  -
(3)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

(1)  Trp Met Met Trp Tyr Trp Gly Pro Ser Leu Tyr Asn Ile Leu Ser      210
(2)   -   -   -   -   -   -   -   -   -   -  Ser  -  Val  -
(3)   -   -   -   -   -   -   -   -   -   -  Ser  -   -   -

(1)  Pro Phe Leu Pro Leu Leu Pro Ile Phe Phe Cys Leu Trp Val Tyr
(2)   -   - Ile  -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

(1)  Ile
(2)   -
(3)   -
```

Fig. 3. Comparison of the amino acid sequences of HBsAg deduced from the nucleotide sequences of the different subtypes of HBV. (1) subtype adr, (2) subtype adw, (3) subtype ayw(8). The bars indicate the same amino acid as in lane (1).

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(1)  Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Ser Val Glu Leu
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -   -   -   -   -  Thr  -   -   -

(1)  Leu Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Ile Arg Asp Leu        30
(2)   -   -   -   -   -   -   -   -   -   -   -  Val  -   -   -
(3)   -   -   -   -   -   -   -   -   -   -   -  Val  -   -   -

(1)  Leu Asp Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

(1)  Glu His Cys Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu        60
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

(1)  Cys Trp Gly Glu Leu Met Asn Leu Ala Thr Trp Val Gly Ser Asn
(2)   -   -   -   -   -   - Thr  -   -   -   -   -   - Asn  -
(3)   -   -   -   -   -   - Thr  -   -   -   -   -   - Val  -

(1)  Leu Glu Asp Pro Ala Ser Arg Glu Leu Val Val Ser Tyr Val Asn        90
(2)   - Gln  -   -   -   -   - Asp  -   -   - Asn  -   -   -
(3)   -   -   -   -   -   -   - Asp  -   -   -   -   -   -   -

(1)  Val Asn Met Gly Leu Lys Ile Arg Gln Leu Leu Trp Phe His Ile
(2)  Thr  -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)  Thr  -   -   -   -   - Phe  -   -   -   -   -   -   -   -

(1)  Ser Cys Leu Thr Phe Gly Arg Glu Thr Val Leu Glu Tyr Leu Val       120
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -   -   -   - Ile  -   -   -   -

(1)  Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala Tyr Arg Pro Pro
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

(1)  Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr Val Val Arg       150
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

(1)  Arg  /   /  Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro
(2)   - Arg Asp  -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   /   /   -   -   -   -   -   -   -   -   -   -   -   -

(1)  Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser       180
(2)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
(3)   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

(1)  Arg Glu Ser Gln Cys
(2)   -   -   -   -   -
(3)   -   -   -   -   -
```

Fig. 4. Comparison of the amino acid sequences of HBcAg deduced from the nucleotide sequences of the different subtypes of HBV. (1) subtype adr, (2) subtype adw, (3) subtype ayw(8). The bars indicate the same amino acid in lane (1) and the oblique lines indicate deletions.

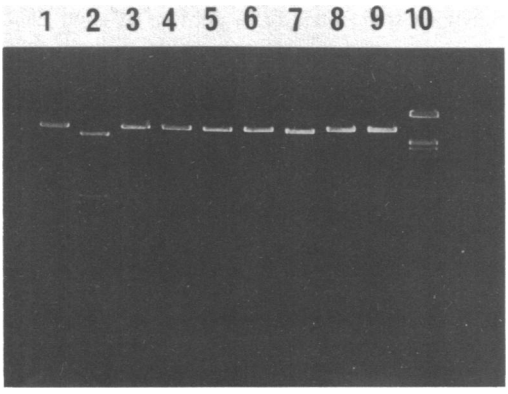

Fig. 5. Restriction fragment analysis of pHBr series . Each recombinant plasmid DNA was prepared by alkaline SDS procedures(19), digested with several restriction endonuclease without isolation of each insert DNA, and electrophoresed on 1% agarose gel. (1) pHBr329, EcoR1 digestion, (2) pHBr330, EcoR1 digestion, (3) pHBr347, EcoR1 digestion, (4) pHBr329, Xho1 digestion, (5) pHBr330,Xho1 digestion, (6) pHBr347, Xho1 digestion, (7) pHBr329, HindIII digestion, (8) pHBr330, HindIII digestion, (9) pHBr347, HindIII digestion, (10) size marker, lamda DNA EcoR1 and HindIII digestion.

DISCUSSION

In this paper, we have reported that the two HBV DNA prepared from the Dane particles with defined subtypes, adr and adw, have been cloned in E. coli., and the complete nucleotide sequences have been determined. The insert of pHBr330(adr) was 3188 nucleotides long, and that of pHBV933 was 3200 nucleotides long, respectively. The length of these cloned viral genomes was different from that of the reported one previously(8). These length differences are due to deletion or additions at two positions in both HBV subtype genomes (Fig.2). The significance of these deletion and additions within the different subtype genomes is not clear, but since they are multiples of three, the reading frame is the same in all four variants (adw, adw2, adr, and ayw).

Because of inadequate information on viral proteins and messenger RNA, it is difficult to analyze the genetic organization solely from the nucleotide sequence. Nevertheless, the coding region for viral proteins could be predicted by searching for the open regions. Galibert et al.(8) found four relatively long open regions in the ayw HBV genome (termed by Tiollais et al.(32) gene S, gene C, gene P,and gene X) and predicted that gene S, gene C, and gene P coded for HBsAg, HBcAg, and DNA polymerase, respectively. These predictions were almost correct in the case of HBsAg and HBcAg genes, because these gene products expressed in yeast (26) or in E. coli. (27,28,31) were biologically active. However, as information on the viral DNA polymerase is limited, the hypothesis that gene P codes for DNA polymerase has not been confirmed. Though we

found the longest open region that codes for a peptide of about 90000 daltons in the adr HBV genome as in the ayw HBV genome, we could not find this long open region in the adw HBV genome, because a stop codon of TAA appears in the center of the region. This may suggest that the splicing system of viral mRNA exist if this adw HBV genome can replicate in vivo or in vitro. Analysis of viral mRNA is necessary for understanding more precisely the genetic organization.

Recently, Pourcel et al.(29) have detected the 2300 base viral specific RNA species in mouse L cell line that was co-transformed with cloned HBV DNA and the herpes simplex virus thymidine kinase gene, and they also have detected a similar RNA in a human hepatoma cell line PLC/PRF/5 that produced HBsAg. Moreover, they mapped this RNA in the viral genome, and predicted the existence of pre-HBsAg, because the initiation codon ATG was located 489bp upstream from the initiation codon of the mature HBsAg in the same reading frame, and a TATA box (30)-like sequence (TATATAA) was also found 72bp upstream from the first ATG of the pre-S region. This open region and the TATA box like sequence (2772-2778) are also conserved in our HBV DNA sequences. It is suggested, therefore, that this sequence is related to the HBsAg promoter.

We found heterogeneity of the HBV genome within the same subtype(Fig.5). Siddiqui et al. also have shown that within the same subtype restriction patterns are slightly different (4). These findings suggest that there is a significant genetic heterogeneity of the HBV genome within the same subtype, although we can not exclude the possibility that these differences reflect artifacts on the cloning procedures.

To understand the biology of HBV, it is necessary to establish a viral infection system and to express the viral specific proteins in cultured cells. We believe that our cloned HBV DNAs are suitable for this purpose.

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