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Hepatitis B virus (HBV) particles are produced in a cell culture system by transient expression of transfected HBV DNA

(DNA transfection/hepatitis B virus replication/pregenome RNA/*in vitro* mutagenesis)

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ABSTRACT **An *in vitro* system for the production of hepatitis B virus (HBV) particles was established by the transient expression of transfected HBV DNA using a human hepatocellular carcinoma cell, HuH-7, as a recipient. The 3.6- and 2.2-kilobase transcripts observed were similar to those in virus-infected liver cells. Both transcripts revealed the microheterogeneity of their 5′ ends. The formation of virus-related particles subsequent to the RNA transcription was demonstrated. The core particles observed in the cytoplasm and the virus particles secreted in the culture medium contained the replicative intermediates of HBV DNA and banded at densities of 1.35–1.36 g/cm^3 and 1.22–1.24 g/cm^3, respectively. Furthermore, the *in vitro* mutagenesis of the template HBV DNA demonstrated that the *P* gene as well as the *C* gene products were essential for the production of HBV particles.**

Infection of hepatitis B virus (HBV) causes acute and chronic hepatitis. Furthermore, HBV is known to be a major cause of human liver cancer. HBV DNA has the following unique structural characteristics (1). Two linear DNA strands (plus and minus) form a circular molecule by annealing each cohesive end region. The minus strand is 3.2 kilobases (kb) in size with a protein linked to its 5′ end. The plus strand usually shows various degrees of incompleteness. Similar HBV-like animal viruses have been discovered in woodchucks (woodchuck hepatitis virus), ground squirrels (ground squirrel hepatitis virus, GSHV), and ducks (duck hepatitis B virus, DHBV). The replication cycle of the virus was proposed by Summers and Mason based on their observations of the *in vivo* replication of DHBV (2). However, the restricted host range of HBV and lack of a cell culture system for virus production have been major problems hindering from understanding of HBV replication and transcription at the molecular level.

In this investigation, an *in vitro* system for the production of HBV particles was established by the transient expression of transfected HBV DNA, using a human hepatocellular carcinoma cell as a recipient. The RNA transcription of HBV DNA, similar to that in virus infection *in vivo*, was observed. Furthermore, mutagenesis of the template HBV DNA indicated virus production to be absolutely dependent on the template plasmid and the *P* gene as well as the *C* gene products to be essential for the synthesis of HBV DNA.

MATERIALS AND METHODS

Cell Lines and DNAs. HuH-7 (3), huH2-2 (4), and HLEC-1 (5) were derived from human hepatocellular carcinomas. HuH-7 and HLEC-1 were negative for HBV infection and also for the integrated HBV DNA. HepG2 (6) was derived from hepatoblastoma and was negative for HBV integration. HuL-1 (7) was an established cell line derived from human fetal liver.

Cloned HBV DNA (pHBV1-1) used for the construction of two different plasmids (Fig. 1) and DNA transfection was from the subtype adr virions, whose nucleotide sequence has been published (8). The first plasmid, pHBV-dimer, contains tandemly repeated sequences of 3.2-kb *Bam*HI-digested whole genome at the *Bam*HI site of pBR322. The second plasmid, pHBV-2, was derived from pHBVX-1, in which a 0.87-kb *Stu* I/*Bgl* II fragment of HBV DNA was inserted into the *Bam*HI site of pBR322 using a *Bgl* II linker DNA. The 3.2-kb *Bam*HI fragment of the HBV genome was then inserted into the *Bam*HI site of pHBVX-1 in the same orientation as that of the insert of pHBVX-1, resulting in the 4.1-kb insert of HBV DNA with 0.87-kb terminal repeats including the cohesive end region. Various mutant plasmids were prepared from pHBV-2 by the insertion of 8-base-pair (bp) *Sac* I linker DNA into the sites of appropriate restriction enzymes, which causes frameshift mutations in the coding frames.

DNA Transfection. DNA transfection was carried out by the calcium phosphate precipitation method (9) with 10 μg of the plasmid of HBV DNA. The cells were plated at a density of 3–5 × 10^6 per 100-mm dish and incubated with DNA precipitates for 6 hr at 37°C.

Preparation of RNA and RNA Transfer Blot Analysis. The total RNA of HBV DNA-transfected cells was prepared by the guanidinium/cesium chloride method (10) 2–3 days following DNA transfection. After electrophoresis in a formaldehyde/agarose gel, RNA was transferred to a nitrocellulose filter (11). A ^{32}P-labeled HBV DNA probe was made by nick-translation (12).

Preparation of Core and Virus Particles. For preparation of core particles, cells were collected 5 days after DNA transfection and suspended in a hypotonic buffer (20 mM Tris·HCl, pH 7.5/50 mM NaCl/5 mM $MgCl_2$/0.1% 2-mercaptoethanol/0.5 mM phenylmethylsulfonyl fluoride). After homogenization, 0.2 vol of 40% sucrose was added to the homogenate. The nuclei and mitochondrial fractions were then pelleted by differential centrifugation. The supernatant fraction was loaded onto a 30% sucrose solution in TNE buffer (20 mM Tris·HCl, pH 7.5/0.15 M NaCl/1 mM EDTA) and centrifuged at 35,000 rpm for 16 hr in a Beckman SW 50.1 rotor. The pellet was subjected to CsCl density gradient centrifugation (1.1–1.6 g/cm^3) at 35,000 rpm for 18 hr. After fractionation, each fraction was assayed for HBV core antigen/HBV e antigen (HBcAg/HBeAg) using the Abbott HBe RIA kit.

Virus particles secreted in the culture medium were concentrated and loaded onto 20% sucrose in TNE buffer and centrifuged at 30,000 rpm for 16 hr. The precipitated particle

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Abbreviations: HBV, hepatitis B virus; HBsAg, HBV surface antigen; HBcAg, HBV core antigen; HBeAg, HBV e antigen; GSHV, ground squirrel hepatitis virus.

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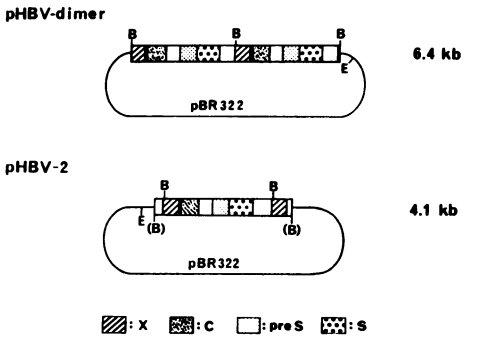

FIG. 1. Structure of template plasmids. The boxed region represents HBV sequences. The sizes of the HBV inserts are indicated on the right. The gene arrangement of HBV is represented by X, C, preS, and S. B, *Bam*HI; E, *Eco*RI; (B), *Bam*HI site of pBR322, where a *Bgl* II-digested HBV fragment was ligated.

fractions were subjected to CsCl density gradient centrifugation, as described above. The assay for HBV surface antigen (HBsAg) was conducted with the Abbott AUSRIA II kit.

For detection of HBV DNA, an aliquot of each fraction was treated overnight with 1% $NaDodSO_4$ and 2 mg of proteinase K per ml at 37°C and then directly subjected to 1% agarose gel electrophoresis. After the Southern transfer of DNA to the nitrocellulose filter (13), the filter was hybridized with a ^{32}P-labeled HBV DNA.

S1 Nuclease Mapping. Appropriate restriction fragments, prepared from the cloned HBV DNA (pHBV1-1), were labeled at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase and then digested with a secondary restriction enzyme to obtain the 5′ end-labeled probe. S1 nuclease digestion was performed as described (14). The protected products were subjected to 7 M urea/polyacrylamide gel electrophoresis. The size of each protected product was estimated by a comparison with the sequence ladder of each probe DNA, which was generated by the method of Maxam and Gilbert (15).

RESULTS

Cell Lines for the Transient Expression System and Specific Transcripts. As for the human liver cells, three hepatocellular carcinoma cell lines and one hepatoblastoma cell line, HuH-7, huH2-2, HLEC-1, and HepG2, respectively, and the human fetal liver cell line HuL-1 were examined. When the plasmid pHBV-dimer was used as a template, the extent of transient expression was assayed by specific RNA transcription of transfected HBV sequences with S1 nuclease mapping. The results indicated only HuH-7 and HepG2 to significantly exhibit the specific transcripts of HBV DNA. The efficiency was higher in HuH-7 than HepG2. In the case of huH2-2, the efficiency was very low. No specific transcription of HBV DNA was observed in other cell lines under the conditions used. These results indicate cell specificity for the transcription of transfected HBV sequences among human liver-derived cell lines. Consequently, the HuH-7 cell line was chosen for the subsequent experiments.

When HuH-7 cells were transfected with pHBV-dimer or pHBV-2, transfer blot hybridization of total RNA revealed the presence of two major transcripts, whose sizes were estimated to be 3.6 kb and 2.2 kb (data not shown). To locate the starting site of each transcript, an S1 nuclease mapping experiment was carried out (Fig. 2*A*). First, a 3.18-kb *Bam*HI/*Sac* II fragment labeled at the site of *Bam*HI was used as a probe and the product was analyzed by alkaline agarose gel electrophoresis. Two DNA fragments, 2.85 kb and 1.4 kb, were mainly protected by RNA transcripts. When

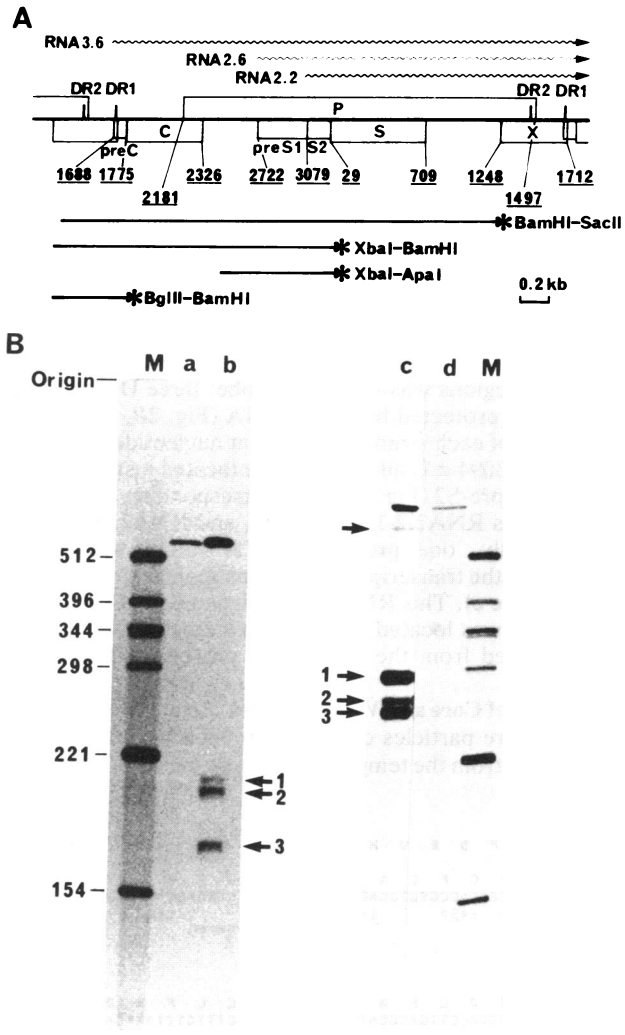

FIG. 2. Mapping of the 5′ ends of major transcripts by S1 nuclease analysis. (*A*) Schematic representation of HBV genome (subtype adr). The viral open reading frames are given in boxes and the nucleotide numbers at both ends of each reading frame are indicated according to the nucleotide sequences of subtype adr (8). The probes used for S1 nuclease analysis are depicted as thick lines, and the ^{32}P-labeled 5′ ends are indicated as asterisks. The structures of major RNA transcripts inferred from the data are indicated as wavy lines. (*B*) Polyacrylamide gel electrophoresis of the protected products. The fragments of 0.58-kb *Bgl* II/*Bam*HI and 0.86-kb *Xba* I/*Apa* I were used as probes and the protected products were electrophoresed through a 6% polyacrylamide gel containing 7 M urea. The 0.58-kb *Bgl* II/*Bam*HI fragment was used in lanes a and b. Lane a, without RNA; lane b, with RNA. The 0.86-kb *Xba* I/*Apa* I fragment was used in lanes c and d. Lane c, with RNA; lane d, without RNA. Arrows indicate the positions of the protected products. Lane M shows the size markers (in nucleotides) of the 5′ end-labeled pBR322 *Hin*fI fragments.

a 2.05-kb *Xba* I/*Bam*HI fragment was used, 1.65-kb and 0.25-kb fragments were protected. From the data, the major transcription appeared to start at two different loci: one in a cohesive end region and the other just upstream from the pre-S2 region.

To determine the precise locations of both initiation sites, a short DNA probe of a 0.58-kb *Bgl* II/*Bam*HI fragment or a 0.86-kb *Xba* I/*Apa* I fragment was prepared and used for S1 nuclease analyses. When a 0.58-kb *Bgl* II/*Bam*HI fragment covering the cohesive end region of HBV DNA was used, three species of protected DNAs were separated (Fig. 2*B*, lanes a and b). The sizes of these protected strands were determined by a comparison with the sequence ladder of the

probe DNA itself. Based on these data, the end of each protected strand is reproducibly located at nucleotides 1657/8, 1664 ± 1, and 1691 ± 1 in the HBV DNA of adr subtype (Fig. 3*A*). Thus, 3.6-kb RNA transcripts have microheterogeneity at their 5′ ends. The transcription of three RNA species, tentatively designated as RNA3.6-1, RNA3.6-2, and RNA3.6-3, appeared to start in the cohesive end region. The 3′ end of 3.6-kb RNA was deduced from the size determined from the electrophoretic mobility of the RNA and the S1 nuclease mapping data of its 5′ end. These results are consistent with the idea that the transcription terminates at the downstream of the poly(A) signal (TATAAA from nucleotide 1790), as indicated by others (17–19).

When a 0.86-kb *Xba* I/*Apa* I fragment containing pre-S1 and pre-S2 regions was used as a probe, three DNA strands were mainly protected by 2.2-kb RNA (Fig. 2*B*, lanes c and d). The end of each strand is located at nucleotides 3063 ± 1, 3081/4, and 3094 ±1, all of which are located just around the first ATG of pre-S2 (Fig. 3*B*). The corresponding RNAs were designated as RNA2.2-1, RNA2.2-2, and RNA2.2-3.

Additionally, one protected DNA strand was weakly observed by the transcript from the upstream region of pre-S1 (Fig. 2*B*, lane c). This RNA was designated as RNA2.6, and its start site was located at the downstream of the "TATA" box estimated from the size of the protected DNA strand (Fig. 3*B*).

Detection of Core and Virus Particles. An attempt was made to detect core particles containing replicative intermediates synthesized from the template of pregenome RNA. A fraction was prepared from the cytoplasm of HuH-7 cells cultured for 5 days following pHBV-dimer or pHBV-2 DNA transfection and subjected to CsCl density gradient centrifugation. After fractionation of the gradient, each fraction was assayed to detect HBcAg/HBeAg specific for the core particles. The presence of replicative intermediate DNAs in each fraction was also analyzed by Southern blot hybridization. As a result (Fig. 4*A*), HBcAg/HBeAg and replicative intermediates of HBV DNAs were detected in fractions 18–21. The density was estimated to be about 1.35–1.36 g/cm^3, which exactly corresponds to that of a DNA-containing viral capsid observed in HBV chronically infected liver (20). In addition, a sharp peak of HBcAg/HBeAg was observed for fraction 22, but it was not correlated with the hybridization pattern of HBV DNA. Thus, HBcAg/HBeAg aggregates may also be present in the cytoplasm.

To ascertain the production of virus-like particles, the particulate fraction prepared from the culture medium was subjected to CsCl density gradient centrifugation (Fig. 4*B*). After fractionation of the gradient, hybridization of HBV DNA to blotted DNA from the fractions showed the hybridizable DNA to band at a density of 1.22–1.24 g/cm^3. The immunological assay of each fraction of HBsAg also indicated association of HBsAg with the DNA band at a density of 1.22–1.24 g/cm^3, clearly indicating the presence of virus particles (20). The major peak of HBsAg was observed at a density of 1.20 g/cm^3, which corresponds to that of small HBsAg particles.

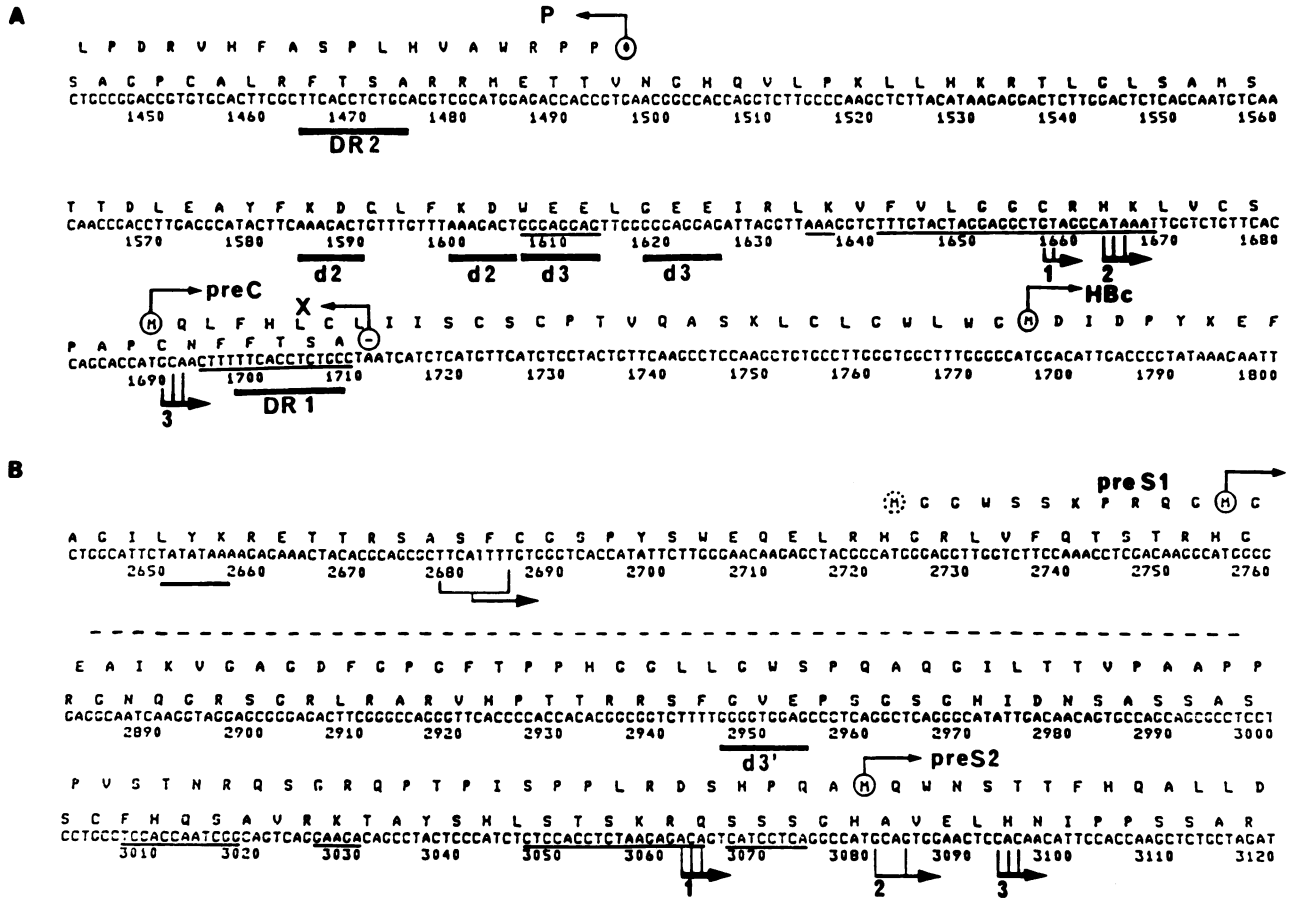

FIG. 3. Nucleotide positions of the 5′ ends of major transcripts. (*A*) The 5′ ends of the 3.6-kb transcripts. (*B*) The 5′ ends of the 2.2-kb and 2.6-kb transcripts. The products protected from S1 nuclease digestion (as shown in Fig. 2) were electrophoresed in a sequencing gel and the size of each strand was estimated by a comparison with the sequence ladder of the probe DNA strand. The thick bars represent directly repeating sequences. In addition to DR1 and DR2, d2 and d3 were identified in the cohesive end region (8), and d3′, which is almost identical to d3, was found in the upstream region of pre-S2. The solid lines under nucleotides indicate the conserved sequence stretches around the initiation sites of RNA transcription between HBV and GSHV (8, 16).

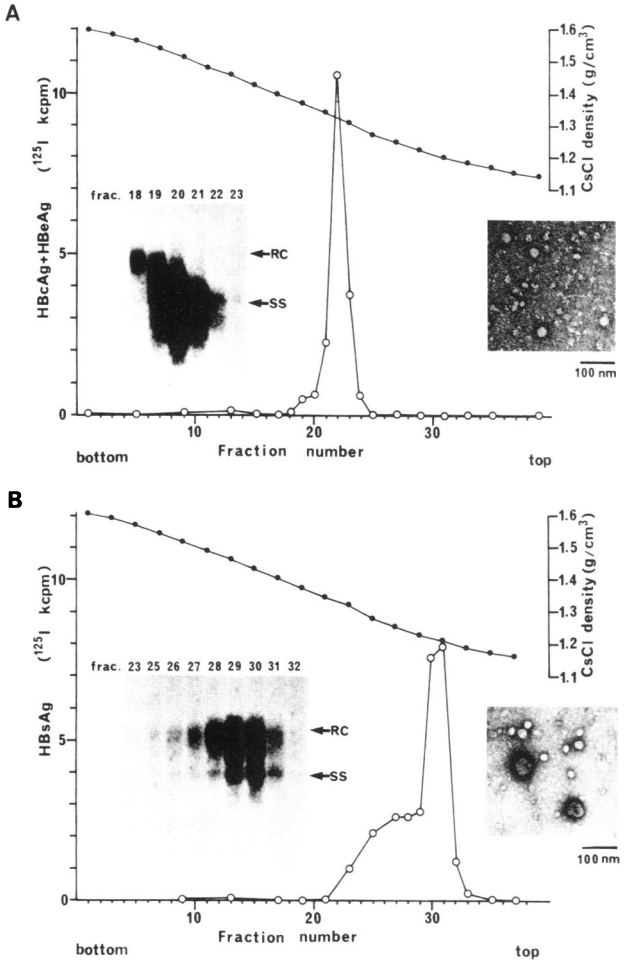

FIG. 4. Analyses of core and virus particles by CsCl density gradient centrifugation. (*A*) Analysis of core particle fractions prepared from the cytoplasm of pHBV-dimer DNA-transfected cells. Open circles indicate the activity of HBcAg + HBeAg detected with the Abbott HBe RIA kit. (*B*) Analysis of virus particle fractions secreted into the culture medium of the pHBV-dimer DNA-transfected cells. Open circles indicate the activity of HBsAg assayed with the Abbott AUSRIA II kit. Closed circles show the density of each fraction. The results of Southern hybridization and of electron microscopy are presented in the *Inset* photographs. RC and SS indicate the positions of relaxed circular DNA and accumulated single-stranded DNA, respectively.

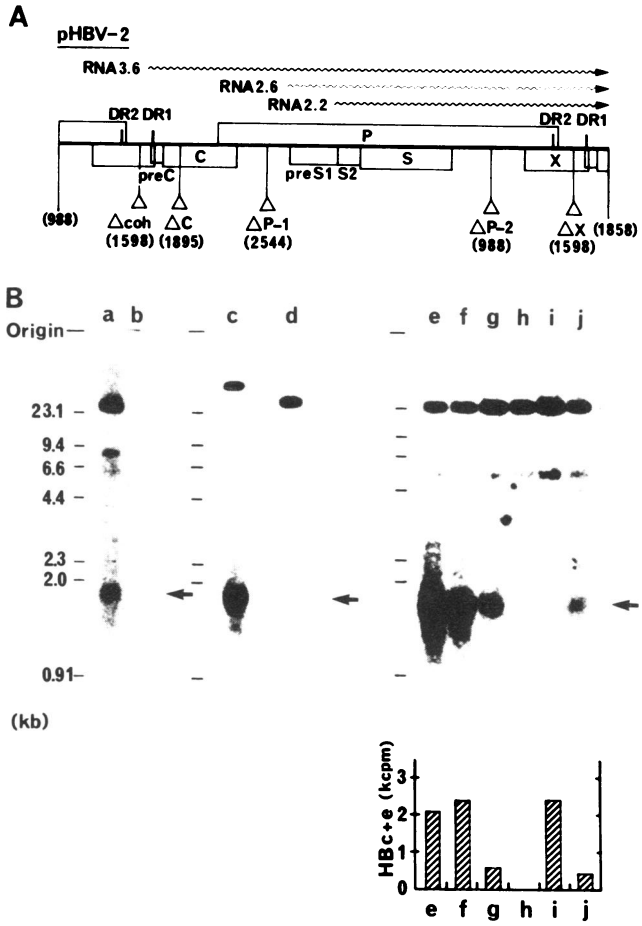

FIG. 5. Analyses of mutant plasmids for the production of viral core particles. (*A*) Location of site-specific mutations on the template plasmid pHBV-2. The site for the insertion of 8-bp linker DNA and designation of each mutant are indicated. (*B*) Assay of mutant templates for the production of DNA-containing core particles. Core fractions were treated with $NaDodSO_4$ and proteinase K, subjected to agarose gel electrophoresis, and blotted on a nitrocellulose filter. HBV DNA was detected by a ^{32}P-labeled probe. The fractions from lanes e–j were also assayed for the amounts of HBcAg and HBeAg. Ten micrograms of each template plasmid per plate [ϕ (diameter), 100 mm] was used. Lane a, pHBV-2; lane b, mock-transfected (without DNA); lane c, pHBV-dimer; lane d, pHBV-2ΔP1; lane e, pHBV-2; lane f, pHBV-2ΔX; lane g, pHBV-2Δcoh; lane h, pHBV-2ΔC; lane i, pHBV-2ΔP2; lane j, pHBV-2ΔC (5 μg) + pHBV-2ΔP1 (5 μg). Arrows indicate the positions of accumulated single-stranded DNA in core particles.

Core and virus particle fractions obtained from each respective peak were examined by electron microscopy (Fig. 4 *A* and *B*), and the diameters were found to be about 27 nm for core particles and about 42 nm for virus particles together with small HBsAg particles. Those observations confirm that DNA-containing core particles are synthesized in the cytoplasm and that virus particles and small HBsAg particles are secreted into the culture medium under the conditions of this transient system.

Mutagenesis of the Template HBV DNA. To confirm the dependency of virus production on transfected HBV genes, various mutations were introduced into template HBV DNAs and their functions were examined by the production of viral core particles. In this experiment, the second plasmid, pHBV-2, was used as a template and insertion mutagenesis was conducted with an 8-bp linker DNA. This caused frameshift mutations in the coding sequences of HBV DNA (Fig. 5*A*). The plasmids pHBV-2ΔP-1 and pHBV-2ΔP-2 have an 8-bp insertion at the site of *Ssp* I (nucleotide 2544) and *Stu* I (nucleotide 988), respectively. The frameshift mutations caused premature termination of the coding frame in the *P* (possibly reverse transcriptase) gene. In the plasmid pHBV-2ΔC, the linker DNA was inserted into the *Stu* I site (nucleotide 1895) and the coding frame of the HBcAg gene was destroyed. In the plasmid pHBV-2ΔX or pHBV-2Δcoh, the linker DNA became inserted into one of the *Dra* I sites (nucleotide 1598) on the pHBV-2 DNA. These two mutations were separately located on either side of pHBV-2 DNA, since the plasmid pHBV-2 has the same cohesive end region at both ends of the insert, as in the case of retroviruses in terminal redundancy. The plasmid pHBV-2ΔX produced a defective product of an *X* gene. The mutation of pHBV-2Δcoh is located upstream from the initiation site of the 3.6-kb RNA transcript.

Using these mutated plasmids as templates, core particle fractions were analyzed to detect replicative intermediates by Southern blot hybridization and core protein by an immunological assay of HBcAg/HBeAg (Fig. 5*B*). When pHBV-2ΔP-1 and pHBV-2ΔP-2 were used, no hybridization of HBV DNA was observed. The production of HBcAg/HBeAg in both cases continued to occur (data for pHBV-2ΔP-1 not shown), indicating only core proteins to be synthesized.

Thus, the *P* gene product is essential for HBV DNA synthesis. Transfection of the plasmid pHBV-2ΔC caused neither production of HBcAg/HBeAg nor hybridization of HBV DNA. The absence of hybridization may be explained by the absence of core proteins to package replicative intermediates. When the two mutant plasmids pHBV-2ΔP-1 and pHBV-2ΔC were transfected together, the formation of DNA-containing core particles was recovered, suggesting the complementation of gene products from two mutant plasmids in the transfected cell. However, the possibility of recombination between two transfected DNAs could not be ruled out. Furthermore, the plasmid pHBV-2Δcoh remarkably reduced the production of core particles, as compared with intact pHBV-2. On the contrary, a little reduction of HBV DNA synthesis was observed by the plasmid pHBV-2ΔX. We also constructed similar mutant plasmids, pHBV-dimerΔcoh and pHBV-dimerΔX, in which the same mutations were introduced into pHBV-dimer DNA. The same results were observed with these mutant plasmids (data not shown). The data suggest that the *X* gene product is not related to the HBV DNA synthesis itself in this system but probably is related to the early stage of virus infection *in vivo*.

DISCUSSION

In the present study, we established an *in vitro* system for the production of HBV by the transient expression of transfected HBV DNA, where cloned HBV DNA was directly introduced into hepatocytes to overcome the barrier for virus infection to the cultured cells. Among several cell lines examined so far, HuH-7 exhibited the most efficient transcription of HBV DNA, and the subsequent virus production was demonstrated. A possible explanation for these preferential expressions is that certain factors specific for human hepatocytes may be responsible for the efficiency of the specific transcription of HBV DNA, since there was no difference in the efficiency of transformation with pSV2-neo DNA using these cell lines as recipients.

Analysis of the RNA transcription of HBV DNA revealed the presence of three species of 3.6-kb RNA or 2.2-kb RNA transcripts with microheterogeneity at their 5′ ends. This microheterogeneity at the 5′ end has been observed in GSHV, in which three species of 3.5-kb genomic transcripts and two species of 2.3-kb *S* gene transcripts were detected (19). The coding information of each transcript in our system could not be fully determined, but, by the mapping of the 5′ ends, it became apparent that RNA3.6-1 and RNA3.6-2 contained the coding frames for precore protein, core protein, and the *P* gene product. RNA3.6-3 did not code precore protein. It was also found that RNA2.2-1 could code pre-S2 HBsAg and HBsAg, but RNA2.2-2 and RNA2.2-3 only contained the coding frame for HBsAg. The RNA transcription was, therefore, similar to that of *in vivo* infection (1).

At the present, we have no clear evidence to specify the pregenome RNA in these three 3.6-kb RNA candidates. However, Seeger *et al.* (16) have proposed that the shortest among the three genomic GSHV RNA species was likely to be the pregenomic template for DNA synthesis, based on their results of mapping the 3′ end of minus strand GSHV DNA at the same location as the 5′ end of the shortest RNA. In the case of HBV DNA, Pasek *et al.* (21) have already demonstrated the location of the 3′ end of minus strand HBV DNA at nucleotide 1692 (in the HBV sequences of subtype adr) by molecular cloning. Therefore, RNA3.6-3 is likely the pregenome RNA, since the 5′ end of RNA 3.6-3 was located at nucleotide 1691 ± 1.

In the case of GSHV, three 5′ ends of 3.5-kb RNA transcripts are distributed over a 30-nucleotide region (19). Similarly, the 5′ ends of 3.6-kb RNA transcripts obtained in the transient expression system were distributed in the region from nucleotide 1655 and 1690. As shown in Fig. 3*A*, a comparison of nucleotide sequences in these two regions indicated several sequence stretches to be conserved in HBV and GSHV (8, 22). Since no particular promoter sequence has been assigned so far for the 3.6-kb RNA transcription, these stretches may play an important role in the initiation of the pregenomic RNA transcription. In the region just upstream from the first ATG of pre-S2, some sequence stretches were also conserved (Fig. 3*B*). Those stretches contain the region homologous to the sequences responsible for the initiation of late gene transcription in simian virus 40 (17). In the upstream region of these sequences, no significant homology could be found between HBV and GSHV.

The presence of core particles in the cytoplasm and secretion of virus particles into the culture medium were demonstrated in the cell culture system by CsCl density gradient centrifugation and Southern blot hybridization (Fig. 4 *A* and *B*). Virus particles collected from the culture medium were distributed in a rather broader range of the CsCl density than core particles. Consequently, virus particles secreted into the culture medium may be coated with surface protein (HBsAg) to various extents. The infectivity of these secreted virus particles remains for future investigation. The present transient expression system coupled with *in vitro* mutagenesis of template HBV DNAs should provide additional information on the molecular mechanism of HBV replication in the future.

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