Infectious hepatitis B virus from cloned DNA of known nucleotide sequence
(chimpanzee transfection/recombinant hepatitis B virus DNA/risk assessment/hepatitis B disease)

HANS WILL†, ROBERTO CATTANEO†, GHALAMREZA DARAI†, FRITZ DEINHARDT†, HUUB SCHELLEKEN§, AND HEINZ SCHALLER†

†Institute of Microbiology, University of Heidelberg, and Zentrum für Molekulare Biologie, Im Neuenheimer Feld 230, 69 Heidelberg, Federal Republic of Germany; Institute for Medical Virology, University of Heidelberg, Im Neuenheimer Feld 324, 69 Heidelberg, Federal Republic of Germany; §Max von Pettenkofer Institute, University of Munich, Pettenkoferstrasse 9a, 8000 Munich 2, Federal Republic of Germany; and †Pri mate Center, Organisation of Health Research, Klei wag 151, 2280 Rijswijk, The Netherlands

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ABSTRACT The infectivity of cloned hepatitis B viral DNA (HBV) has been tested in chimpanzees to identify a fully functional HBV genome and to assess the risk associated with its handling. Only one of two HBV DNA sequence variants tested was shown to be infectious. “Clone purified” virus of predicted nucleotide sequence was produced from the infectious HBV DNA, and the cloned viral genome was identical in structure with naturally occurring HBV. Infection could be initiated independent of whether circular monomeric or plasmid integrated dimeric forms of the viral genome were inoculated, but the infectivity of the DNA depended on liver cell transfection or intrahepatic injection. Intravenous injection of high doses of infectious HBV DNA did not induce hepatitis, suggesting that there is virtually no risk associated with routine laboratory handling of cloned HBV DNA.

A functional analysis of the hepatitis B virus (HBV) genome is hampered by the narrow host range of the virus (man and chimpanzees) and by the lack of a system for propagation in vitro. In addition, cloned HBV DNA molecules are heterogeneous both in size, due to small insertions and deletions, and in nucleotide sequence (up to 12% (1), and some of these may be defective. This heterogeneity is related not only to subtype specificity but is also observed in different clones of the same serotype or even those obtained from a single patient (2, 3).

As a first step in identifying an infectious HBV genome of known sequence, we recently showed that hepatitis can be induced in chimpanzees by inoculation with cloned double-stranded HBV DNA (4). However, to limit the number of test animals in that pilot experiment, a mixture of three different cloned HBV DNA species in different physical forms was used, and several routes of inoculation were simultaneously employed. In the present study, we have analyzed these parameters separately. As a result, we describe here a simplified experimental procedure for successful infection with cloned HBV DNA. In addition, a homogeneous virus stock was obtained from a cloned HBV DNA of known nucleotide sequence.

MATERIALS AND METHODS
Cloned HBV DNA and Plasmids Used for Inoculation.
Circular double-stranded HBV-2 DNA was isolated from plasmid pTKH10, which consists of a full-length HBV genome (5) inserted into vector pAGO (6), and circular HBV-14 DNA from plasmid pHBV14-1 (2, 7), respectively, by digestion with EcoRI or PstI, separation by low melting point agarose gel electrophoresis, isolation of the linear HBV DNA genome fragments, and ligation with T4 ligase under conditions of preferential circularization (8). A plasmid (pTKHH2) containing two HBV-2 genomes in a tandem head-to-tail arrangement was constructed by partial digestion of plasmid pTKH10 with EcoRI and religation with a 10-fold excess of linear HBV-2 genomes isolated from plasmid pTKH10.

Restriction Analysis of “Clone Purified” Viruses. Virus particles from the sera of chimpanzees were pelleted through a sucrose gradient, and the DNA was 32P-labeled by the endogenous polymerase reaction (9).

The virus was treated at 37°C for 2 hr with protease K (2 mg/ml) in 1% NaDodSO4/10 mM EDTA/50 mM sodium acetate, extracted twice with phenol and once with chloroform/isoamyl alcohol (24:1), and sized on a 1% agarose gel. Alternatively, the virus was resuspended in 40 mM Tris hydroxyacetate, pH 7.5/5 mM sodium acetate/1 mM EDTA/0.1% NaDodSO4 and analyzed on a 1% agarose gel as described (10). Completion of restriction enzyme digestion was monitored by the presence of plasmid carrier DNA.

RESULTS
To define the conditions needed for successful transfection of chimpanzee liver cells, we first varied the DNA sequence type [HBV-2 (5) and HBV-14 (7)] and the physical state (circles and tandemly linked plasmid integrated) of the cloned HBV DNA (Table 1). In this experiment, three chimpanzees (chimpanzees 1, 2, and 3) were inoculated by the same route (reinjection of liver cells previously transfected in vitro). Second, three other chimpanzees (chimpanzees 4, 5, and 6) were inoculated by intrahepatic (i.h.) or i.v. injections of cloned HBV DNA. Third, two chimpanzees were inoculated intravenously with serum containing virus produced in chimpanzees who had been inoculated with cloned HBV DNA (chimpanzees 6* and 7) and one chimpanzee (chimpanzee 5*) inoculated with HBV-containing serum of a human patient.

Hepatitis B Induction by Cloned HBV DNA. Based on serological and biochemical assays, a typical picture of a self-limited hepatitis B was observed ≈10 weeks after inoculation in three of six animals (Fig. 1 A–C). As a first serological marker, HBV surface antigen (HBsAg) was detected in the serum. Two to 3 weeks later, a second HBV-specific antigen, HB e antigen (HBeAg), was detected, indicating the acute phase of hepatitis B infection. This was also confirmed by histological examination of liver biopsies showing mild hepatic lesions as described (13), and

Abbreviations: HBV, hepatitis B virus; i.h., intrahepatic; HBsAg, HBeAg, surface antigen, core, and e antigen, respectively, of HBV; kb, kilobase(s); bp, base pair(s).

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increased alanine aminotransferase levels were also observed as expected (data not shown). None of the three animals developed a chronic form of the disease, and all viral antigens and HBV DNA disappeared 25-40 weeks after inoculation and were replaced by antibodies to HBV-specific antigens. In all three successfully transfected animals (chimpanzees 1, 3, and 4), the same HBV DNA sequence (HBV-2) had been used, demonstrating that this DNA was a functional HBV genome and that i.h. injection was sufficient (chimpanzees 4) to initiate a replication cycle with cloned HBV DNA in liver cells. Both physical forms of the DNA tested, circular (chimpanzees 1 and 4) and a tandemly linked version (chimpanzee 3), were infectious. In contrast, inoculation of HBV-14 DNA did not induce any signs of infection during an observation period of 5 months (chimpanzee 2), even though a transfection procedure had been used that had

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### Table 1. Experimental outline of inoculations

<table>
<thead>
<tr>
<th>Chimpanzee</th>
<th>Route of inoculation</th>
<th>Type of HBV DNA</th>
<th>Physical form of HBV DNA</th>
<th>Hepatitis B induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Transfection</td>
<td>2</td>
<td>Circular</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>Transfection</td>
<td>14</td>
<td>Circular</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>Transfection</td>
<td>2</td>
<td>Tandem</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>i.h. injection</td>
<td>2</td>
<td>Circular</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>i.v. injection</td>
<td>2</td>
<td>Tandem</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>i.v. injection</td>
<td>2 + 14</td>
<td>Circular and tandem</td>
<td>No</td>
</tr>
<tr>
<td>5*</td>
<td>Injection</td>
<td>Unknown</td>
<td>Virus (human serum)</td>
<td>Yes</td>
</tr>
<tr>
<td>6*</td>
<td>Injection</td>
<td>2</td>
<td>Virus</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>Injection</td>
<td>2</td>
<td>Virus</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Chimpanzees 1 and 2 were inoculated with 10 µg of closed HBV DNA circles and chimpanzee 3 was inoculated with 10 µg of plasmid pTKHH2 by liver cell transfection as described (4). Chimpanzee 4 was inoculated by i.h. of 10 µg of closed-circular HBV-2 DNA [dissolved in 1 ml of 1× RPMI-1640 medium (Flow Laboratories), containing 50 µg of DEAE dextran]. Plasmid pTKHH2 (10 mg) dissolved in the same medium was injected intravenously into chimpanzee 5. Chimpanzee 6 was inoculated 3 times intravenously with cloned HBV DNA, first with 80 µg of the inoculum used in our pilot experiment (4), after 15 weeks with 1 mg of a mixture of closed-circular and plasmid-integrated (pTKHH2) HBV-2 DNA (0.1 mg and 0.9 mg, respectively), and after a further 6 months, with 10 mg of plasmid pTKHH2 DNA. Two years after intravenous inoculation with cloned DNA, chimpanzee 5 (renamed chimpanzee 5*) was infected with 0.1 ml of a HBV-containing serum from a human patient, chimpanzee 6 (renamed chimpanzee 6*) was infected with 0.5 ml of serum taken from a chimpanzee during the acute stage of infection induced by inoculation with three cloned HBV DNAs (4), and chimpanzee 7 was infected with 0.5 ml of a corresponding serum taken from chimpanzee 1 containing virus induced by cloned HBV-2 DNA alone.

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**Fig. 1.** Time course of serological HBV markers and viral DNA detected in sera of three chimpanzees after inoculation with cloned HBV DNA (A, B, and C) and of one chimpanzee infected with clone-purified HBV-2 (D). HBV DNA was determined by DNA dot blot hybridization (11) and the serological markers were from commercially available enzyme linked- or radioimmunoassays (12). Low values in the various assays (1 pg of HBV DNA per ml of serum; a ratio of <3 of bound radioactivity of sample/negative control for HBeAg and HBsAg; a titer of <200 for anti-HBsAg, anti-HBcAg, and anti-HBeAg) are indicated by dashed lines.
been successful with HBV-2 DNA (chimpanzees 1 and 3). This suggests that HBV-14 DNA represents a defective HBV genome. Further evidence for this interpretation comes from a successful challenge experiment carried out with chimpanzee 6*, infected with serum obtained during the acute stage of infection from a chimpanzee used in our pilot experiment [chimpanzee Corrie (4)]. In this experiment, the major species of cloned DNA used for inoculation was HBV-14 with only little HBV-2 DNA, but the restriction map of the genome of the clone-purified virus, which could be established only after amplification by passage (chimpanzee 5*; also, see below), was that of HBV-2 but not HBV-14 (data not shown). This again demonstrates the viability of the HBV-2 genome and confirms the interpretation that HBV-14 is a defective genome.

Negative results were obtained with the two chimpanzees (chimpanzees 5 and 6) inoculated intravenously and several times (chimpanzee 6) with large amounts of cloned HBV-2 DNA (~10 mg) of proven infectivity after i.h. inoculation. After an observation period of 2 years, both animals were challenged by i.v. inoculation with live virus. Chimpanzee 5 (renamed chimpanzee 5*) was challenged with HBV-containing serum from a human patient and chimpanzee 6* was challenged with virus produced from cloned DNA (see above). Starting ~4 weeks thereafter, all serological signs of a typical self-limited hepatitis B were observed (data not shown), demonstrating their susceptibility to HBV infection.

**Detection and Characterization of Clone-Purified HBV from Serum and Liver.** To prove directly the presence of viral particles, the sera and liver biopsies from the transfected animals were analyzed by DNA hybridization using 32P-labeled HBV DNA as a probe. As predicted from the serological data, sera from chimpanzees 1, 3, and 4 were positive for HBV DNA at the acute stage of HBV infection (Fig. 1 A–C), indicating that viral particles had been produced from cloned HBV DNA. We next asked whether the corresponding HBV DNA also had the characteristic structure of naturally occurring HBV genomes encapsidated in virions (1). Virus pelleted from the sera was subjected to the endogenous polymerase reaction and the 32P-labeled DNA was analyzed by agarose gel electrophoresis. Two DNA bands migrating with an apparent size of 3.2 and ~4.0 kilobases (kb), corresponding to the position of linear and open circular HBV DNA, were observed with viruses from all three animals (data shown for chimpanzee 1; Fig. 2A, lane +, after proteinase K digestion; lane –, without proteinase K digestion). Without proteinase K digestion, both bands migrated slightly slower, indicating the presence of a genome-linked protein. Since similar observations are made with naturally occurring HBV (14, 15), three of the main characteristics of HBV virions are also present in the virus formed after transfection: a virion-associated polymerase, a genome-linked protein, and a partially single-stranded genome. Size and location of the single-stranded region and a restriction map of the viral genome were determined by restriction enzyme analysis of the endogenously labeled HBV DNA. As shown in Fig. 2B, the viral genomes from the serum from chimpanzee 1 digested with several restriction enzymes yielded a DNA fragment pattern as predicted for cloned HBV-2 DNA labeled in the gap region (Fig. 3). Some minor bands (asterisks) fit the prediction that they are derived from linearized genomes as has also been reported for duck HBV and ground squirrel HBV (16, 17). These results demonstrate that a virus indistinguishable in genome structure from naturally occurring HBV had been produced from cloned HBV-2 DNA.

To characterize HBV-specific DNA molecules present in acutely infected liver of our test animals and to compare it with replication intermediates described for naturally in

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**Fig. 2.** Restriction enzyme analysis of viral genome isolated from serum of chimpanzee 1 after 32P-labeling using the virion-associated polymerase and sizing on agarose gels. (A) Genome after (+) and prior to (−) proteinase K digestion. (B) After proteinase K digestion and cleavage with several restriction enzymes. Some minor bands derived from linearized genomes are marked by asterisks; oc, open circular form of the genome; lin, linearized genome.

**Fig. 3.** Restriction map of HBV-2 genome. In inner two circles, nick (arrow) and gap region (asterisks) of viral genome are indicated. In outer circles, only those fragments (length in bp) overlapping gap region are shown.
was also observed when strand-specific probes were used (data not shown).

All these data provide further evidence that cloned HBV DNA had initiated a normal HBV infection. The unusual pattern of replicative intermediates seen in our pilot experiment (4) was only found in a restricted area of the liver (possibly the injection site), whereas it was normal in another piece of liver (data not shown). This suggests that at the site of inoculation of DNA, some abnormal replicative intermediates can accumulate, but normal virus is also produced and is subsequently infecting adjacent liver tissue.

Passage of the Clone-Purified HBV-2. All serological, histopathological, and biochemical data strongly suggested that the clone-purified virus produced in chimpanzees by transfection of liver cells was identical in structure and infectivity to naturally occurring HBV. For a direct proof of its infectivity, another animal (chimpanzee 7) was inoculated intravenously with serum taken at the acute phase of HBV infection from chimpanzee 1. Two weeks after inoculation, HBsAg was already detected in the serum of this animal as the first serological sign of hepatitis B infection (Fig. 1D), and a typically self-limited hepatitis B was observed during the following weeks, demonstrating that infectious viral particles had been produced and secreted into the serum of the transfected chimpanzee. The virus titer of this serum was ~10 times higher than that obtained from transfected animals (data not shown), and this allowed us to characterize the clone-purified virus in more detail by restriction enzyme digestion of the 32P-labeled genome as described above. Using rarely cutting restriction enzymes (Fig. 5) and a set of multcutting enzymes (data not shown) covering 66 and 136 base pairs (bp), respectively, of the HBV-2 sequence, we found the same restriction enzyme cleavage pattern as predicted from the HBV-2 DNA sequence (compare Figs. 3 and 5). This confirms the conclusion that infectious HBV had been produced from the cloned HBV-2 DNA. The size analysis of the replicative DNA intermediates in the liver of this animal (21) is also consistent with this statement.

**DISCUSSION**

We have demonstrated that a cloned double-stranded HBV DNA of known nucleotide sequence (HBV-2) is infectious after i.h. injection independent of whether it is in a covalently closed circular form or tandemly linked and integrated into a plasmid. This result confirms and extends our pilot experiment described previously (4). The long-term study described here revealed that the infectivity of cloned HBV genomes depended on the isolate, the route of inoculation, and less, if at all, on the physical state of the DNA. From the two HBV DNA sequence variants tested, only one isolate (HBV-2 DNA) was infectious, and the virus produced was shown to possess a nucleotide sequence identical or similar to that of the transfecting DNA. Thus, we have produced a high-titered virus stock of defined nucleotide sequence and of proven infectivity.

The restriction analysis of the 32P-labeled viral genomes from our virus stocks demonstrates that within the sensitivity of our assay no major HBV sequence variants were present. These virus stocks can be regarded as optimally defined reference inocula to be used to further study the rate of DNA and protein sequence divergence during several passages in the animal. Another important by-product of our experiments consisted of obtaining HBV-infected liver tissue containing HBV-specific transcripts of defined nucleotide sequence that are required for their unambiguous mapping (21, 22). In contrast to cloned HBV-2 DNA, the second isolate (HBV-14 DNA) induced no signs of hepatitis B in a chimpanzee inoculated only with this DNA, and furthermore, a virus of the corresponding sequence was not produced in another chimpanzee [Corrie (4)] transfected by a mixture of small amounts of HBV-2 and large amounts of HBV-14 DNA simultaneously, as shown by the challenge experiment performed with chimpanzee 6*. Both experiments strongly suggest that HBV-14 is a defective viral genome. Considering the nucleotide sequence divergence between various cloned HBV DNAs (1), it is conceivable that defects may also be present in other cloned HBV genomes. Consequently, conclusions drawn from comparative sequence studies are of limited value, until the infectiv-
ity of the corresponding clones has been established.

The sequence change(s) leading to the defect in cloned HBV-14 DNA cannot be localized with certainty. HBV-14 DNA differs from HBV-2 DNA in 117 bp and by a 6-bp duplication at the end of the X reading frame [nucleotide position 3103 (7)]. Since this region of the HBV genome is also thought to be involved in the initiation of its replication, it is conceivable that it is the 6-bp duplication that renders the HBV-14 genome defective. Another possible locus of the defect is a base change at nucleotide position 3184 (7), creating a stop codon and thereby eliminating a continuous "pre-C" open reading frame present in four other cloned and sequenced HBV genomes (1). However, this base change is less likely to make HBV-14 defective, because six other DNA clones isolated from the same serum (2) all contained the same base change at this site. Furthermore, an independently cloned HBV DNA of different serotype (3) also lacks a continuous "pre-C" open reading frame. Whether the defective HBV-14 genome was created in vivo or might result from the cloning procedure as described for ground squirrel HBV (17) remains unclear.

There was no notable difference whether we used covalently linked circular or tandemly linked plasmid integrated DNA: both were infectious and both forms of the DNA can probably serve as templates to produce all transcripts required for the HBV replication cycle. Alternatively, genome-sized circular HBV DNA molecules without plasmid sequences could also be produced by an excision and ligation process from the plasmid integrated form. If such mechanisms were essential for the infectivity of integrated HBV DNA, templates substantially shorter than a dimer would be sufficient to initiate HBV replication, and similar mechanisms for the activation of chromosomally integrated HBV DNA in vivo could be envisaged.

The route of inoculation was important for successful induction of HBV with cloned DNA. Intravenous injection was ineffective even though one chimpanzee was inoculated 3 times at intervals using an =1000-fold higher dose of HBV DNA (total, 11 mg) of proven infectivity. This indicates that the risk associated with handling small amounts (pg to ng) of cloned HBV DNA, as is done in a growing number of clinical laboratories, is very low or nonexistent. Our data also suggest that HBV DNA, possibly present in minute amounts in currently available or in future vaccines, presents, if at all, a minimal risk as long as it is not encapsidated in virions. This risk may be even less for the partially single-stranded HBV genomes isolated from the virions.

Finally, our results demonstrate that it is possible in principle to perform a functional analysis of the HBV genome by cloning in *Escherichia coli* and testing it in vivo in chimpanzees. This approach can be extended to the related animal hepatitis viruses (16).

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