Trans-complementation of the C gene of human and the P gene of woodchuck hepadnaviruses

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A 5 bp insertion was introduced into the BstEII site at nucleotide 2815 in DNA of hepatitis B virus (HBV) and a mutant HBV genome was produced, which coded for envelope and core proteins, but not for DNA polymerase, due to a frameshift. Cultured hepatoma cells (HepG2) were simultaneously transfected with a plasmid harbouring a tandem dimer of the mutant HBV DNA and another plasmid harbouring a tandem dimer of DNA of woodchuck hepatitis virus or duck hepatitis B virus. The replication of mutant HBV DNA, incapable of encoding DNA polymerase, was accomplished by cotransfecting woodchuck hepatitis virus DNA, but not by duck hepatitis B virus DNA. These results indicated a trans-complementation of the C and P genes in mammalian hepadnaviruses beyond a species barrier.

Hepadnaviruses are small, encapsidated DNA viruses with a genome consisting of a circular, partially double-stranded DNA molecule and include human hepatitis B virus (HBV), woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV), duck hepatitis B virus (DHBV) and heron hepatitis B virus (HHBV) (Summers et al., 1978; Marion et al., 1980; Mason et al., 1980; Feitelson et al., 1986; Sprengel et al., 1988). They have in common a narrow host range and a restricted organ tropism for the liver. Their genetic organizations are quite similar with three open reading frames in different phases (Tiollais et al., 1985). They are the S gene coding for surface or envelope protein, the C gene coding for core or nucleocapsid protein and the P gene coding for a putative DNA polymerase. In addition, mammalian hepadnaviruses have the X gene, coding for a protein with a transcriptional trans-activating function (Twu & Schloemer, 1987; Spandau & Lee, 1988).

The close similarity of mammalian hepadnaviruses is revealed by a shared antigenicity of envelope as well as core protein in HBV and WHV (Werner et al., 1979; Cote & Gerin, 1983). However, it is not yet known whether or not the DNA polymerase is replaceable in these two mammalian hepadnaviruses infecting different species.

Avian hepadnaviruses, in contrast, appear to be very different from mammalian hepadnaviruses. The envelope protein of DHBV does not cross-react with that of HBV or WHV (Cote & Gerin, 1983), which is attributable to homology in the entire nucleotide sequence of only 40% between HBV and DHBV, much less than that of 60% between HBV and WHV (Kodama et al., 1985).

Trans-complementation of the C and P genes of DHBV, recently reported by two independent groups (Chang et al., 1989; Schlicht et al., 1989), has led us to evaluate the capacity of DNA polymerases of heterologous hepadnaviruses to replicate a P gene defective mutant of HBV DNA in an in vitro transient expression system.

HBV DNA clone (pPYW310; 3182 bp, subtype ayw), was isolated from serum of a Papua New Guinean blood donor, provided by Dr D. G. Woodfield of the New Zealand Red Cross Blood Centre. WHV DNA clone (WH81; 3320 bp) was a gift from Dr J. L. Gerin of the Georgetown University Medical Center; its sequence was determined by Kodama et al. (1985). DHBV DNA (pDHB101; 3021 bp) was propagated from duck serum donated by Dr J. Summers of the Institute for Cancer Research, Fox Chase Center.

The complete HBV DNA clone (pPYW310) was cleaved with BsrEII (New England Biolabs). Sticky ends were blunted with the Klenow fragment (Takara Biochemicals, Kyoto, Japan) and ligated. HBV DNA thus obtained was used to transform Escherichia coli strain DH1 (Hanahan, 1983). A 5 bp insertion, generated at nucleotide (nt) 2815 by this procedure, induced a frameshift in the P gene (Fig. 1). The mutant, HBV DNA (ΔP), had the S gene coding for the envelope protein and the C gene for the core protein, but could not encode DNA polymerase due to a frameshift in the P gene. To prepare another mutant, pPYW310 was
cleaved at the unique SacII site (nt 2001) and subjected to digestion with S1 nuclease (Takara Biochemicals). Blunt ends were ligated, which resulted in a 2 bp deletion (Fig. 1). The mutant, HBV DNA (AC), had a defect in the C gene, but the S and P genes were preserved. The desired frameshifts in the mutants were verified by determining their nucleotide sequences (Sanger et al., 1977).

Two copies of wild-type HBV DNA (pPYW310), HBV DNA (ΔP) or HBV DNA (AC), connected in a head-to-tail arrangement, were inserted into the PstI site of plasmid vector pSP65 (Melton et al., 1984). The tandem dimers of WH81 and pDHB101 were inserted into EcoRI and BamHI sites, respectively. They were introduced into HepG2 cells (Aden et al., 1979) by the calcium phosphate transfection method (Spandidos & Wilkie, 1984). Subconfluent HepG2 cells (1 × 10⁶) were added to a 25 cm² Petri dish (Miles Laboratories) and grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% foetal calf serum (Flow Laboratories), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM-L-glutamine at 37 °C in an atmosphere containing 5% CO₂. After 24 h each dish received 12.5 μg of recombinant DNA (6.25 μg each of two different DNA preparations in cotransfection experiments) in a mixture of 225 μl of 1 mM-Tris–HCl pH 8.0 supplemented with 0.1 mM-EDTA, 25 μl of 2.5 mM-CaCl₂ and 250 μl of HEPES buffer (50 mM, pH 7.1) made to 280 mM with NaCl and 15 mM with Na₃HPO₄ (total volume 500 μl). The medium was replaced after 16 h of culture and every 2 days thereafter.

Medium maintaining HepG2 cells (2 to 8 ml) was harvested after 2 to 7 days of culture and centrifuged in a Beckman SW60 rotor at 50000 r.p.m. for 1 h. The pellet was washed and dissolved in 250 μl of 1 x SSC (150 mM-NaCl, 15 mM-sodium citrate, pH 7-0) containing 1 mM-EDTA. To the solution were added 25 μl of 10% SDS and 25 μl of Pronase E (20 mg/ml). DNA was extracted with phenol–chloroform, precipitated with ethanol and then dissolved in 10 mM-Tris–HCl pH 8.0 containing 1 mM-EDTA. DNA thus prepared was subjected to electrophoresis on 1% agarose, transferred to a nitrocellulose filter membrane and analysed by the method of Southern (1975).

Hybridization was carried out at 42 °C for 36 h in a solution containing 50% formamide, 6 x SSC, 0.5% SDS, 10 mM-EDTA, 5 x Denhardt’s solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone and 0.1% bovine serum albumin) along with 100 μg/ml denatured salmon sperm DNA. Probes were cloned HBV DNA, WHV DNA and DHBV DNA, labelled with [α-32P]dCTP (Amersham) by the multiprime labelling system (Feinberg & Vogelstein, 1983). Also used were HBV RNA probes specific for either the minus or the plus strand and labelled with [α-32P]dCTP (Amersham) by the SP6 polymerase vector system (Melton et al., 1984). After hybridization the filter membrane was rinsed with 0.1 x SSC containing 0.1% SDS at 65 °C for 3 h with the renewal of washing solution every 30 min. Annealings and washes were sufficiently stringent to prevent cross-species hybridization.

Virus particles secreted from HepG2 cells transfected with plasmids carrying hepadnavirus DNA of various species, either alone or in combination, were analysed by Southern blot hybridization (Table 1). With the conditions used for hybridization the radiolabelled probes detected HBV DNA, WHV DNA and DHBV DNA in a species-specific manner (Fig. 2). Input DNA used for transfection did not contribute to the signal on the blots. Replicative intermediate forms of hepadnavirus DNA were demonstrated, thereby attesting to the replication of HBV, WHV and DHBV in transfected HepG2 cells.
Table 1. DNA in virus particles secreted from HepG2 cells transfected with plasmids harbouring DNA of various hepadnaviruses or defective mutants of HBV DNA, either alone or in combination

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<tr>
<th>HepG2 cells transfected with plasmids harbouring a tandem dimer of</th>
<th>DNA in virus particles hybridizable with</th>
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<td>HBV [[^{32}\text{P}]\text{DNA}</td>
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<td>HBV DNA</td>
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<td>WHV DNA</td>
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<td>DHBV DNA</td>
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<td>HBV DNA ((\Delta P))</td>
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<td>HBV DNA ((\Delta C))</td>
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<td>HBV DNA ((\Delta P)) and HBV DNA ((\Delta C))</td>
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<td>HBV DNA ((\Delta P)) and WHV DNA</td>
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<td>HBV DNA ((\Delta P)) and DHBV DNA</td>
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<td>HBV DNA ((\Delta C)) and WHV DNA</td>
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<td>HBV DNA ((\Delta C)) and DHBV DNA</td>
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* HepG2 cells were transfected with plasmids harbouring a tandem dimer of one or other of the HBV DNA clones, its mutant defective in the P gene (\(\Delta P\)) or C gene (\(\Delta C\)), WHV DNA and DHBV DNA clone. HepG2 cells were also transfected with combinations of two plasmids harbouring different hepadnavirus DNA clones.

Neither HBV DNA (\(\Delta P\)) nor HBV DNA (\(\Delta C\)) could replicate by itself, but they complemented each other for the production of virus particles containing HBV DNA. The synthesis of HBV DNA (\(\Delta P\)) was complemented by cotransfecting WHV DNA, but not by cotransfecting DHBV DNA (Fig. 2). The replication of HBV DNA (\(\Delta C\)) was not complemented by either WHV or DHBV.

HBV DNAs in virus particles secreted from HepG2 cells cotransfected with HBV DNA (\(\Delta P\)) and WHV DNA were minus strands; they hybridized with the RNA probe specific for minus-strand HBV DNA, but not with that specific for plus-strand HBV DNA (Fig. 3, lane 2). In contrast, both minus- and plus-strand HBV DNAs were detected in virus particles secreted from HepG2 cells cotransfected with HBV DNA (\(\Delta P\)) and HBV DNA (\(\Delta C\)) (Fig. 3, lane 1) or from those transfected with wild-type HBV DNA (Fig. 2a, lane 1).

The most remarkable characteristic of hepadnaviruses is their replication by the reverse transcription of an RNA pregenome, in a manner similar to retroviruses (Summers & Mason, 1982). However, the expression strategy of the gene coding for polymerase is quite different in these two groups of viruses. The pol gene of retroviruses is expressed as a nucleocapsid–polymerase (gag–pol) fusion protein by ribosomal frameshifting during the translation of overlapping genes (Jacks & Varmus, 1985). The P gene of hepadnaviruses, in contrast, is expressed independently of the C gene by an internal initiation (Chang et al., 1989; Schlicht et al., 1989). As a consequence trans-complementation is achieved by a C gene defective mutant and a P gene defective mutant, for both HBV (Yaginuma et al., 1987) and DHBV (Chang et al., 1989; Schlicht et al., 1989). We have observed such trans-complementation between mammalian hepadnaviruses of different species, but not between mammalian and avian hepadnaviruses.

The hepadnavirus DNA contains two direct repeats (DR1 and DR2), which are considered important in

![Fig. 3. HBV DNA in virus particles secreted from HepG2 cells.](image-url)
initiating the synthesis of both plus- and minus-strand DNAs (Seeger et al., 1986; Lien et al., 1987; Will et al., 1987). The amino-terminal domain of the P gene encodes the terminal protein, which is believed to prime the reverse transcription of pregenome RNA, within DR1, to initiate the production of a minus-strand HBV DNA (Bartenschlager & Schaller, 1988). It may also be involved in the primer switch from DR1 to DR2 to initiate the production of a plus-strand HBV DNA for the conversion from a linear to a circular genome conformation.

DR1 and DR2 of mammalian hepadnaviruses are composed of 11 nt, with a single difference at the eighth nt of C for HBV and G for WHV, as well as GSHV (Galibert et al., 1982; Seeger et al., 1984; Kodama et al., 1985; Cohen et al., 1988; Girones et al., 1989). However, the two direct repeats of mammalian hepadnaviruses are quite different from those of avian hepadnaviruses. DR1 and DR2 of both DHBV and HHBV are composed of 12 nt, five of which are different from those of HBV. Due to this difference of DR1/DR2 the terminal protein of DHBV would not be able to bind with the HBV pregenome. In contrast, a minor difference in the HBV DR1/DR2 might enable binding of the terminal protein of WHV. We would like to propose the compatibility of HBV DR1/DR2 with the primer of WHV, but not with that of DHBV, to explain our results of the complementation tests.

The DNA polymerase of the WHV we used (WH81) did not work as well as that of HBV for the replication of HBV DNA. HBV DNAs synthesized by WHV DNA polymerase were minus-strand; double-stranded HBV DNAs in relaxed-circular and linear forms were not detectable. However, the observed low efficiency in plus-strand synthesis may not be ascribable to species specificity. The WH81 clone had a 3 bp deletion in the coding region for terminal protein, incapable of encoding glycine at the 15th residue from the N terminus. In actuality little replication of plus-strand WHV DNAs was observed in HepG2 cells transfected with WH81 (data not shown), which might be ascribed to this defect.

Another result of the complementation test that deserves attention is the lack of HBV DNA in virus particles secreted from HepG2 cells cotransfected with the HBV DNA (AC) mutant and WHV DNA. The terminal redundant region of the RNA pregenome is important for packaging of the viral RNA pregenome (Enders et al., 1987). It is tempting to speculate that the WHV core protein would not be able to package the HBV pregenome–WHV polymerase complex owing to incompatibility of the terminal redundant region.

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References


SHORT COMMUNICATION


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