

Short
Communication*In vitro* replication competence of a hepatitis B genotype D/A recombinant virus: dissimilar biological behaviour regarding its parental genotypes

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Hepatitis B virus (HBV) DNA recombinants contribute to ~30% of the overall full-length sequences already deposited in GenBank. However, their biological behaviour has not been analysed so far. In this study, the *in vitro* replication kinetics of the first D/A recombinant from the American continent differed from its parental genotypes, exhibiting higher extracellular levels of HBV DNA and hepatitis B e antigen. Southern blots of intracellular core-associated HBV DNA were in agreement with such results. Because this recombinant was obtained from an Argentinian injecting drug user belonging to a vulnerable community, these results are of singular relevance for regional public health. Further *in vivo* studies are urgently needed to determine the pathogenicity of these replicative competent clones.

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Hepatitis B virus (HBV) infection is one of the most prevalent chronic viral infections among human beings. It often leads to cirrhosis and/or hepatocellular carcinoma, which is annually responsible for 1 million deaths worldwide. As a result, it is considered one of the major world health concerns.

Eight HBV genotypes (HBV/A–HBV/H) have been reported based on a sequence divergence greater than 8% over the entire genome. Another two genotypes

referred to as HBV/I and HBV/J have also been proposed. Genotypes are further subdivided into subgenotypes, which have been recognized in HBV/A–D and F, if the divergence in the whole genome reaches between 4 and 8% (Lin & Kao, 2011). The global impact of HBV recombinants has also been described recently (Shi *et al.*, 2012).

Evidence for the influence of HBV genotypes and/or subgenotypes on the progression of liver diseases in acute, fulminant and chronic infection, the clinical outcome and the response to antiviral treatment have been reported by several researchers (Kramvis & Kew, 2005; Lin & Kao, 2011; Liu *et al.*, 2005). However, information about the effects of recombinant genomes on the clinical, prognostic and therapeutic aspects of the HBV infection is still lacking. Therefore, the aim of this study was to preliminarily analyse the very early replication dynamics of the infection of a HBV D/A recombinant and compare them with those

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Two supplementary figures are available with the online version of this paper.

of its parental genotypes (HBV/D and HBV/A) and of a highly replicative genotype (HBV/C) in an *in vitro* experimental system.

Serum samples were obtained from two previously recruited subjects (Trinks *et al.*, 2008): (i) H-IDU6 who was chronically infected with an HBV/D3 genome, as determined by partial S and pre-C/C phylogenetic analysis (PHYLIP package version 3.5c: Joseph Felsenstein, University of Washington, Seattle, WA, USA); and (ii) H-IDU7 who showed a HBV 'false' occult infection (Raimondo *et al.*, 2008) by a D3/A2 recombinant genome (breakpoints at nt 147 and 636, according to *EcoRI* restriction site numbering), as characterized by full-length phylogenetic and Simplot version 3.5.1 (Stuart Ray, John Hopkins University, Baltimore, MD, USA) analysis. Accordingly, this strain exhibited a recombinant HBV/A2 DNA region which corresponded to nt 147–636 of the S gene inserted in a backbone corresponding to HBV/D3.

HBV DNA was extracted from serum using QIAamp DNA blood kits (Qiagen). First, in order to fully characterize H-IDU6 HBV DNA, the complete genome was amplified and analysed by a reported method (Trinks *et al.*, 2008). Then, pUC19 plasmids deprived of promoters (Invitrogen) carrying a 1.24-fold HBV genome of each sample were constructed as described previously (Sugiyama *et al.*, 2006). Plasmids for HBV/A2 and HBV/C (Sugiyama *et al.*, 2006) were also included in this study.

After 24 h of culture, Huh7 cells were transfected with plasmids equivalent to 24 µg HBV DNA constructs using Lipofectamine 2000 transfection reagent (Invitrogen). Transfection efficiency was monitored by GFP expression using flow cytometry (BD FACSCanto; BD Biosciences) after cell transfection with a pTARGET (Promega)–GFP expression vector. Except for Southern blotting, all experiments were conducted twice for each clone.

At 24 and 72 h post-transfection (p.t.), hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were determined from the supernatant by ARCHITECT (Abbott). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also measured to determine cell viability. Supernatants from cells treated solely with Lipofectamine were included as negative controls.

At 72 h p.t., in order to measure HBV DNA viral load in supernatants (ruling out extracellular free HBV DNA or HBV RNA), such fluids were ultracentrifuged at 22 000 *g* for 5 min to enrich HBV and HBV core particles; pellets were resuspended and jointly treated with DNase I and RNase A at 37 °C for 3 h (Sugiyama *et al.*, 2006). To confirm the validity of the DNA extraction method (only from virions and capsids, but not from free recombinant plasmid DNA) in the supernatants, Huh7 cells mock transfected with HBV/C plasmid in the absence of Lipofectamine were considered as a further (expected) negative control due to DNase and RNase treatment. HBV load was measured subsequently (COBAS TaqMan HBV Test; Roche).

At 72 h p.t., in order to confirm the HBV replication among all the studied clones, cells were lysed and the density of core-associated HBV DNA was compared by Southern blot hybridization with a mix of full-length probes of each genotype involved in the experiment (A2, C, D3 and D3/A2; Sugiyama *et al.*, 2006).

Student's *t*-test was used to compare the means and SD between any pair of samples: *P*<0.05 was considered statistically significant.

Biochemical and virological features of both patients from whom sera were obtained are shown in Table 1. The full-length genome was amplified from sample H-IDU6 and subjected to phylogenetic analysis. This sample was ascribed to HBV/D3 and the presence of recombination was ruled out by Simplot (Figs S1 and S2b, available in JGV Online). None of the isolates possessed the mutation G1896A, A1762T or G1764A, which could have interfered with the expression of HBeAg and the efficiency of pre-genome encapsidation for replication. As expected for HBV/D, T1858 was observed in both isolates.

For construction of HBV D3/A2 recombinant and HBV/D3 vectors (Sugiyama *et al.*, 2006), at least 25 clones for each PCR-amplified HBV hemigenome [fragments A (nt 17–1799) and B (nt 1595–239); Sugiyama *et al.*, 2006] from each sample were sequenced and phylogenetically analysed. All clones from the HBV/D3 sample were ascribed to the D3 subgenotype. With regard to the D3/A2 recombinant sample, all 25 clones derived from fragment B were ascribed to the D3 subgenotype; in contrast, 40 % of the analysed clones from fragment A were D3/A2 recombinants with breakpoints at nt 147 and 636, 32 % belonged to the A2 subgenotype, 16 % to recombinant clones with breakpoints at nt 505 and 630, 8 % to recombinant clones

Table 1. Biochemical and virological features of patients from whom HBV isolates were recovered

Feature	H-IDU6	H-IDU7
Gender	Male	Male
Age	35	26
HBsAg	+	–*
HBeAg	+	+
Anti-HBc Ab	+	+
Anti-HCV Ab	+	+
Anti-HIV Ab	+	+
HBV viral load	>110 × 10 ⁶ IU ml ⁻¹	>110 × 10 ⁶ IU ml ⁻¹
HBV genotype	D3	D3/A2 recombinant

*T113S and T131N mutants were detected within the major hydrophilic region of the deduced S amino acid sequence. A negative result for HBsAg had been originally obtained with the serum collected in 1995 and then studied with AxSYM (Abbott) (Trinks *et al.*, 2008). These mutants became detectable when supernatants collected from Huh7 transfected cells were tested by means of the ARCHITECT assay (Abbott) in this study.

with breakpoints at nt 519 and 630, and 4% to D3 subgenotype (Fig. 1a). Because the most abundant recombinant clones were those exhibiting breakpoints at nt 147 and 636 (Fig. 1a and Fig. S2a), they were considered representative of the whole viral population and thus selected for D/A replicon construction.

Transfection efficiency ranged from 24.1 to 24.4% and cell viability was similar in all groups ($P>0.05$; data not shown, available upon request).

At 24 h p.t., the D3/A2 clone produced the highest levels of both antigens ($P<0.0001$; Fig. 1b). Moreover, at 72 h p.t., the HBsAg levels from HBV/A2 and the recombinant clone were the highest ($P>0.05$), followed by HBV/C and HBV/D3 ($P<0.0001$; Fig. 1b). At this time point, the recombinant

clone produced the highest levels of HBeAg compared with its parental genotypes and also the HBV/C clone ($P<0.0001$; Fig. 1b).

At 72 h p.t., the HBV/C clone showed the highest viral load in the supernatant, closely followed by the recombinant clone, whose extracellular HBV DNA level was, in turn, higher than those from its parental genotypes ($P>0.05$; Fig. 1c).

Southern blotting undoubtedly confirmed previously published results regarding the HBV/C clone, which exhibited the highest intracellular replication level (Sugiyama *et al.*, 2006). Interestingly, the level of the recombinant was higher than those from its parental genotypes (Fig. 1d). Negative controls processed in parallel confirmed the specificity of the above-mentioned results.

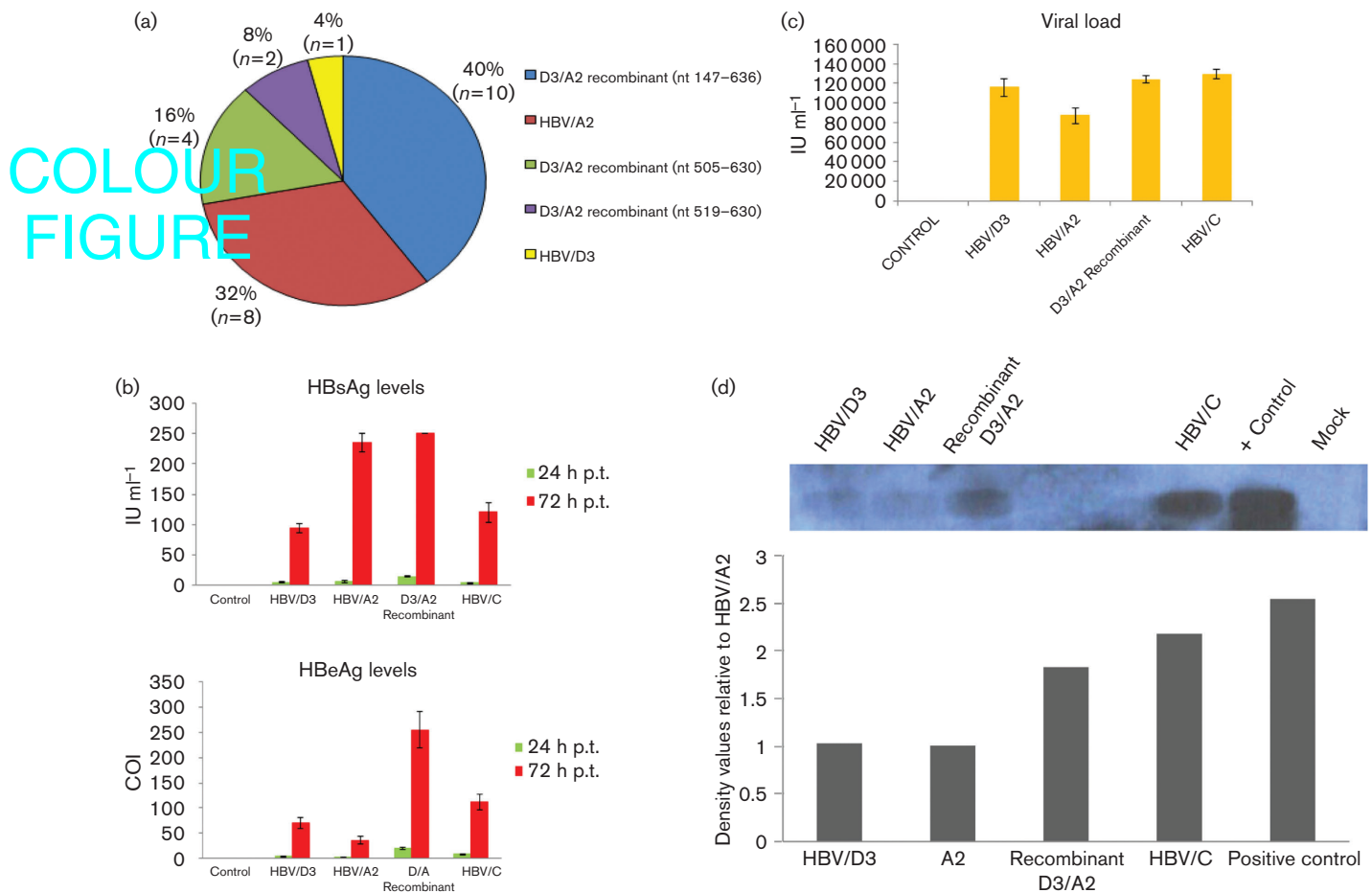


Fig. 1. (a) Analysis of clones derived from fragment A obtained from the recombinant strain H-IDU7. (b) HBsAg and HBeAg extracellular levels. COI, cut-off index. (c) HBV viral load in supernatant. (d) Core-associated HBV DNA in Southern blot analysis of Huh7 cell lysates transfected with plasmid constructs of genotype HBV/D3, HBV/A2, D3/A2 recombinant and HBV/C. An aliquot of non-transfected unlabelled full-length HBV/A2 probe (3.2 kb; positive control) and Huh7 cell lysates treated solely with transfection reagent (mock) were also included. The density of the bands corresponding to a hybridization signal was normalized to that obtained with the A2 clone, which exhibited the lowest density value (density=1). An asterisk represents a statistical difference of $P<0.0001$ when compared with all the remaining genotypes. Double asterisks indicate a statistical difference of $P<0.0001$ in comparison with genotypes HBV/D3 and HBV/C. The absence of asterisks represents no statistical difference.

The influence of genotypes and/or subgenotypes on disease progression and clinical outcome of HBV infection is well documented. However, information regarding HBV recombinant behaviour is unknown.

In a previous study (Trinks *et al.*, 2008), our group isolated a novel intergenotypic D/A recombinant strain from a patient (H-IDU7) co-infected with HIV/HCV. This strain, which was the first full-length D/A recombinant genome characterized from the American continent, exhibits a HBV/D3 backbone genome with an inserted segment of HBV/A2 within the Pol gene. After cloning this sample, we documented the co-circulation of HBV/D3, HBV/A2 genomes together with three different types of D/A recombinants of which one was dominant. In future studies, it will be interesting to compare the biological behaviour of these three dissimilar recombinants in an attempt to elucidate the reason(s) for the observed dominance of the D3/A2 recombinant clones with nt 147–636 breakpoints.

The observation of pure HBV/D3 and HBV/A2 clones confirms that co-infection with different HBV genotype strains is a prerequisite for recombination (Zhou *et al.*, 2012). However, the mechanism of selection of a given strain in mixed infections, i.e. DNA exchange or (less likely throughout the lifespan of a given individual) DNA mutation evolution, still remains unknown.

In this study, the replication kinetics of this recombinant differed from those of its parental genotypes, exhibiting higher extracellular levels of HBV DNA, similar (to A2) or higher (than D3) HBsAg, and higher (than both) HBeAg values. The significance of these findings should be explored by using the primary hepatocyte infection and also *in vivo* uPA-SCID mice models.

Taking into account that one of the HBV DNA-binding sites for CREB transcription factor is placed at nt 143–154 and that it enhances HBsAg expression levels, as previously shown for an A2 replicon (Tacke *et al.*, 2005), it seems plausible that those genomes showing the CCTGTG-ACGAAC binding site would exhibit similarly high

HBsAg expression. This sequence was observed in the recombinant clone, as the 5' breakpoint for the A2 insert is placed at nt 147. Interestingly, such a binding site is mutated in the HBV/D3 replicon (CCTGCGCTGAAC, mutations underlined), which could account for a lower level of pre-S/S transcription efficiency for such a genotype (in contrast to HBV/A2) and consequently for a lower level of HBsAg expression, as reported previously (Sugiyama *et al.*, 2006), the latter result also being observed in our study. Although HBsAg levels frequently reflect intrahepatic HBV replication in WT genomes (Chan *et al.*, 2011), they do not necessarily mirror HBV DNA levels in some mutated pre-S/S genomes (Pollicino *et al.*, 2012). Moreover, it has been shown that HBV/A2 is associated with higher HBsAg secretion and lower DNA replication compared with other genotypes (Sugiyama *et al.*, 2006). Interestingly, our recombinant clone produced high levels of HBsAg, HBeAg and DNA viral load, whose highest titres are usually associated with HBeAg secretion. These results might be explained by the presence of an A2 insert in the PreS2/S region and a D3 backbone in the pre-C/C region.

However, the recombinant exhibited even higher HBeAg values and intracellular HBV DNA levels than the parental D3 clone, which could be explained by the presence of mutations T1766 and A1770 in the D3 backbone of the recombinant D3/A2 clone, which form putative hepatocyte nuclear factors 1 (HNF1)- and HNF3-binding sites related to enhanced viral replication (Baumert *et al.*, 1996; Günther *et al.*, 1996; Fig. 2). Moreover, the single mutation T1664C observed within the core upstream regulatory sequence (CURS; nt 1636–1742) in the recombinant, but which was absent in the D3 parental genotype, might also account for such a difference in HBeAg secretion. As the CURS region exerts a strong stimulating effect on the basal core promoter (Yuh *et al.*, 1992), it is tempting to speculate that such a mutation might produce the higher HBeAg levels observed with the recombinant compared with the D3 parental clone.

Because this recombinant strain was obtained from an intravenous drug user belonging to a highly vulnerable

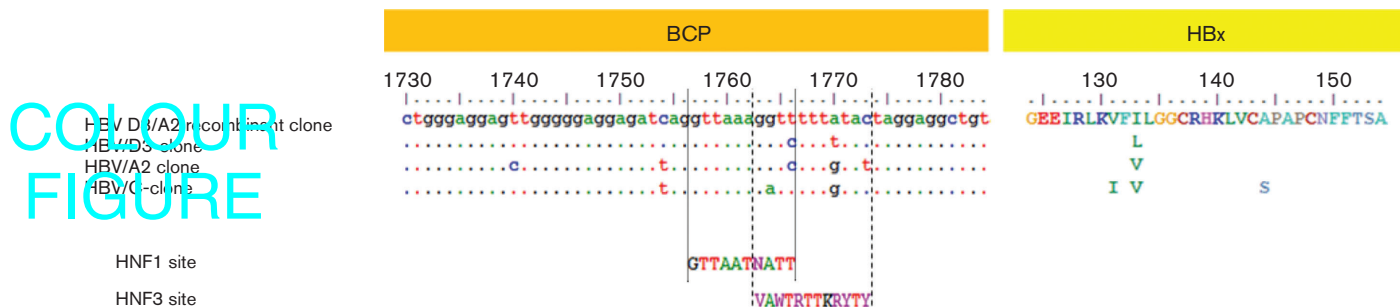


Fig. 2. Sequences of the basal core promoter (BCP) nt 1730–1785 and the overlapping region of the X protein of the HBV clones. The binding sites for HNF1 and HNF3 are aligned with the corresponding region of the HBV genome. The symbols for nucleotide ambiguities are as follows: V, A/C/G; W, A/T; R, A/G; K, G/T; Y, C/T.

group in Argentina, these results are of singular relevance for regional public health. Further *in vivo* studies are needed to determine the pathogenicity of these replicative competent clones.

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