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A duck hepatitis B virus strain with a knockout mutation in the putative X ORF shows similar infectivity and in vivo growth characteristics to wild-type virus

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Abstract

Hepadnaviruses including human hepatitis B virus (HBV) and duck hepatitis B virus (DHBV) express X proteins, HBx and DHBx, respectively. Both HBx and DHBx are transcriptional activators and modulate cellular signaling in in vitro assays. To test whether the DHBx protein plays a role in virus infection, we compared the in vivo infectivity and growth characteristics of a DHBV3 strain with a stop codon in the X-like ORF (DHBV3-X-K.O.) to those of the wild-type DHBV3 strain. Here we report that the two strains showed no significant difference in (i) their ability to induce infection that resulted in stable viraemia measured by serum surface antigen (DHBsAg) and DHBV DNA, and detection of viral proteins and replicative DNA intermediates in the liver; (ii) the rate of spread of infection in liver and extrahepatic sites after low-dose virus inoculation; and (iii) the ability to produce transient or persistent infection under balanced age/dose conditions designed to detect small differences between the strains. Thus, none of the infection parameters assayed were detectably affected by the X-ORF knockout mutation, raising the question whether DHBx expression plays a physiological role during in vivo infection with wild-type DHBV.

Keywords: Hepadnaviridae; DHBV; Duck hepatitis B; HBx; DHBx; X protein; In vivo; Outcome of infection; Rate of spread

Introduction

Hepadnaviruses are small, enveloped DNA viruses containing a partially double-stranded, relaxed circular DNA (rcDNA) genome with a size of 3.0–3.3 kb. Following infection of a host cell, the rcDNA is converted into covalently closed circular DNA (cccDNA) in the nucleus (Tuttleman et al., 1986). This cccDNA is transcribed by the host RNA polymerase II to produce subgenomic mRNAs and a greater-than-genome-length RNA called pregenomic RNA (pgRNA). All hepadnaviruses replicate by reverse transcription of pgRNA into rcDNA. Messenger RNA species encode the envelope proteins, the nucleocapsid or core protein, the polymerase protein, and in mammalian hepadnaviruses, the X protein. HBV and woodchuck hepatitis virus (WHV) both express X proteins, HBx and WHx, respectively, in vivo (Feitelson et al., 1993; Kay et al., 1985), and HBx- and WHx-specific immune responses can be detected in infected individuals (Jacob et al., 1997; Moriarty et al., 1985). A variety of functions have been described for these X proteins using in vitro assays, including transactivation of viral and cellular promoters as well as proapoptotic properties and a possible role in the development of HCC (see Diao et al., 2001, for a review).

Avihepadnaviruses isolated from gray herons, snow geese, a Ross goose, and white storks also have an open reading frame (ORF) in a position similar to that of the X

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gene of mammalian hepadnaviruses (Chang et al., 2001). However, putative X proteins derived from these ORFs are smaller in size, ranging from 66 to 87 amino acids (aa), compared to HBx (154 aa) and WHx (141 aa). It was recently proposed that the smaller size of the avian X proteins has resulted from insertion of stop codons upstream of the X-like ORFs during evolution, thus preventing expression of larger and structured X proteins similar to those in the mammalian hepadnaviruses (Lin and Anderson, 2000). More than 20 DHBV isolates have been described and cluster into the "Chinese" and "Western country" isolates (Triyatni et al., 2001). DHBV strains also contain an X-like ORF similar in length to those of other avihepadnaviruses, but the X-like ORF does not have an AUG start codon. However, an X-like protein has been detected in DHBV-infected liver using antipeptide antibodies; it has been proposed that this may be expressed with a length of up to 114 aa using a nonconventional start codon (Chang et al., 2001).

Little is known about the possible in vivo functions of X proteins. In initial experiments woodchucks transfected with WHV genomes containing a truncated WHx or a WHx-K.O. showed no signs of infection (Chen et al., 1993; Zoulim et al., 1994). However, in more recent in vivo experiments, animals transfected with WHV genomes containing mutations in the WHx ORF demonstrated evidence of low-level WHV infection, with transient viraemia and the development of anti-WHV core and/or anti-WHV surface antibodies (Zhang et al., 2001). In these studies the WHx mutants reverted to wt within 2 weeks of infection, suggesting a selective advantage for wt strains expressing WHx protein and an important role for the X protein in vivo. In transgenic mice carrying an integrated HBV DNA with mutations in the HBx ORF, virus replication and virion export still occurred with comparable HBcAg levels in the liver to those observed in transgenic mice with wt HBV (Reifenberg et al., 2002).

Thus, circumstantial evidence suggests that the X protein may play some important as yet undefined role in the overall process of in vivo infection; however, this has not been correlated with the spectrum of effects described for X protein in vitro. In vivo systems are available for DHBV and can be used to explore the function of X. We therefore wished to test, as a starting point, whether the DHBx protein plays a role in virus infection in vivo by comparing the infectivity and growth characteristics of the wt DHBV3 strain, with the DHBV3-X-K.O. strain containing a stop codon in the putative DHBx-ORF (Chang et al., 2001). Specifically, we aimed to produce infectious stocks of each strain and to compare the strains in terms of their infectious dose, the rate and level of virus spread in vivo, and the ability to cause acute or persistent infections under age/dose-controlled conditions. These results were compared with data from experiments with the Australian strain of DHBV (AusDHBV) (Jilbert et al., 1996, 1998; Triyatni et al., 2001). None of the infection parameters assayed were detectably affected by the X-ORF knockout mutation, raising the question of whether DHBx expression plays a physiological role during in vivo infection with wt DHBV.

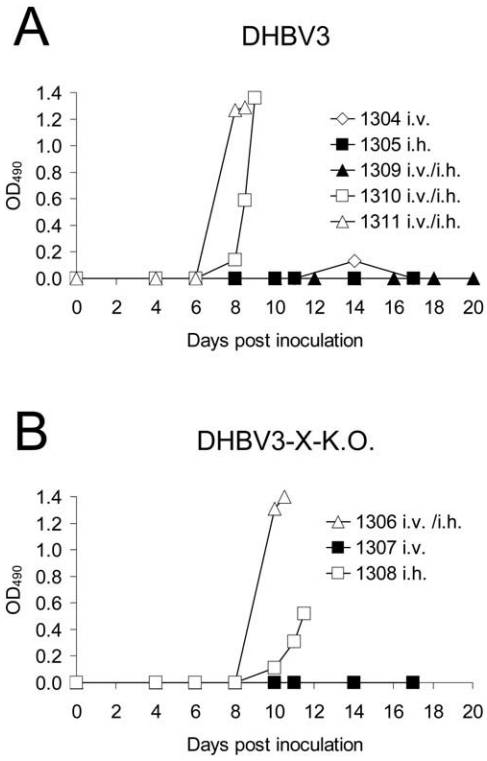

Fig. 1. Development of antigenemia following DHBV DNA transfection. One- to 2-day-old DHBV-negative ducks were inoculated ih and/or iv with 100 μg of plasmid containing a head-to-tail dimer of the genome of DHBV3 (A) or DHBV3-X-K.O. (B) and monitored for serum DHBsAg by ELISA. Serum samples from animals 1311 (DHBV3) and 1306 (DHBV3-X-K.O.) were collected at the time of autopsy and used as infectious viral stocks for future experiments. OD, optical density (490 nm).

Results

Generation and analysis of infectious virus stocks

One- to 2-day-old DHBV-negative ducks were inoculated intrahepatically (ih) and/or intravenously (iv) with 100 μg of plasmid containing a head-to-tail dimer of the genome of DHBV3 or DHBV3-X-K.O. (Chang et al., 2001) and monitored for serum DHBsAg using a standard screening ELISA (Jilbert et al., 1996). Of five ducks inoculated with pDHBV3, two (1305, 1309) remained DHBsAg-negative; one (1304) showed a low and transient viremia at day 14 postinoculation (p.i.) only, and two (1310, 1311) developed significant viraemia by day 8 (Fig. 1A). Of three ducks inoculated with pDHBV3-X-K.O., two (1306, 1308) showed viremia at day 10 p.i. and one (1307) remained DHBV-negative throughout the observation period (Fig. 1B).

Table 1
Comparison of DHBV wt and DHBV3-X-K.O. serum stocks

	DHBV3 wt[a]	DHBV3-X-K.O.[b]	AusDHBV[c]
DHBsAg [μg/ml]	110	140	50
DNA content [vge/ml]	3.1×10^{10}	1.0×10^{10}	2.3×10^{10}[d]
ID_{50}/ml	2.5×10^{9}[e]	3.4×10^{9}[e]	2.8×10^{9}[e]
Ratio IP/vge[f]	1/18	1/4	1/12

[a, b] The serum stocks used were derived from duck 1311 and 1306, respectively.

[c, d] A virus stock was used that was different to the one used in previously published data (Jilbert et al., 1996), and quantitative PCR was used for DHBV DNA determination instead of southern blot hybridisation.

[e] The 95% confidence limits were ± 1.03 $\log_{10}$ units.

[f] IP, infectious particles = $0.67 \times ID_{50}$.

Serum samples from animals 1311 (DHBV3) and 1306 (DHBV3-X-K.O.) were collected at the time of autopsy (days 8 and 10 p.i., respectively) and used as infectious stocks for future experiments. Each of the stocks was tested by quantitative DHBsAg ELISA and found to contain 110 and 140 μg/ml of DHBsAg for DHBV3 and DHBV3-X-K.O., respectively (Table 1). Viral genome equivalents/ml (vge/ml) determined by quantitative PCR were 3.1×10^{10} and 1.0×10^{10} for DHBV3 and DHBV3-X-K.O., respectively. For the determination of the ID_{50} of the viral stocks, 1- to 2-day old ducks were inoculated iv with dilutions of each stock containing the equivalent of 10,000, 1000, 100, 10, 1, and 0.1 vge and then bled twice a week for 4 weeks. Infection was detected by the appearance of DHBsAg in serum and the presence of DHBsAg in autopsy liver sections (days 11–33 p.i.), which gave concordant results. Serum stocks from each strain were found to have similar ID_{50} titers (Reed and Muench, 1938) and had ratios of DNA genomes/infectious particle of 18 and 4 for the DHBV3 and DHBV3-X-K.O. strains, respectively, similar to those observed using pooled serum from ducks congenitally infected with an AusDHBV (Table 1) (Jilbert et al., 1996).

Spread of virus infection following low-dose viral inoculation

One- to 2-day-old DHBV-negative ducks were inoculated iv with 100 μl of diluted duck serum containing 1500 ID_{50} of DHBV3 (1311) and DHBV3-X-K.O. (1306). Each day from days 3 to 14 p.i., two animals from each group were autopsied for detailed examination. No marked pathology was observed in liver sections, including assessment of bile-duct cell proliferation, and fat and glycogen content, and no differences were seen between DHBV3, DHBV3-X-K.O., and uninfected ducks (not shown). DHBsAg-positive hepatocytes were first detected at day 4 p.i. for DHBV3 with 0.04 and 0.08% infected cells (Figs. 2A and B), while with DHBV3-X-K.O. DHBsAg was first seen at day 5 p.i. (0.005 and 0.07% of hepatocytes DHBsAg-positive). From days 4 (DHBV3) or 5 p.i. (DHBV3-X-K.O.) an exponential increase in infected cells was observed up to days 7 to 8 p.i., after which time >95% of hepatocytes remained DHBsAg-positive.

Viral replicative intermediates (RI) including double-stranded linear (dsl), single-stranded (ss), and relaxed circular (rc) DNA were first detected in the liver by Southern blot hybridisation at day 6 p.i. for both strains and peaked at days 8–9 (Fig. 2C). The amount of RI detected corresponded to the percentage of hepatocytes infected, i.e., a slight delay was seen for DHBV3-X-K.O. until day 8 p.i. Western blot analysis from fully infected livers showed two bands at 28 and 36 kDa for DHBsAg (Fernholz et al., 1993) and one band at 32 kDa for DHBcAg (Sprengel et al., 1988). Expression levels of both DHBsAg and DHBcAg were similar for DHBV3 and DHBV3-X-K.O. (Fig. 2D).

DHBsAg was first detected in serum at low levels at day 6 p.i. for two ducks inoculated with DHBV3 and for one duck inoculated with DHBV3-X-K.O. Serum DHBsAg levels in all ducks peaked at around day 9 p.i. and then declined by 0.5–1 log (Fig. 3A). The two strains showed no significant differences in serum DHBsAg content. DHBV DNA was first detected in serum using quantitative PCR at day 3 p.i. for both strains and the levels peaked at day 9 p.i. before declining by 0.5–1 log, consistent with DHBsAg serum levels (Fig. 3B). Again, DHBV3 and DHBV3-X-K.O. showed no significant differences in serum DHBV DNA content.

DHBsAg in extrahepatic tissue was first detected in renal glomeruli at day 7 p.i. and thereafter for both virus strains (data not shown). Detection of DHBsAg in glomeruli has been associated with immune complexes, indicating an early antiviral humoral immune response (Freiman et al., 1988). Staining of DHBsAg in spleen, especially in either peri-ellipsoid cells within the periarteriolar lymphoid sheath and/or in germinal centers, was observed as soon as the liver was fully infected, although not in all animals. Occasional staining of single acinar cells was observed in pancreas from day 10 p.i. onward, which has been shown to involve DHBV replication (Jilbert et al., 1988). No differences regarding infection of extrahepatic tissue were observed between DHBV3- and DHBV3-X-K.O.-inoculated animals.

Age/dose-related outcomes of infection

We have shown before that whether an acute or persistent infection will occur depends on the age of ducks and the dose of the DHBV used in the inoculum (Jilbert et al., 1998). To examine whether expression of DHBx might influence these outcomes, 14-day-old ducks were inoculated with defined doses of the DHBV3 or DHBV3-X-K.O. strains. Serum samples of the inoculated ducks were analyzed for DHBsAg twice a week for 5 to 9 weeks p.i. by ELISA. Ducks that developed persistent infection showed similar liver histology and distribution of DHBsAg in liver and extrahepatic sites to the persistently infected ducks described in the previous section. All animals inoculated

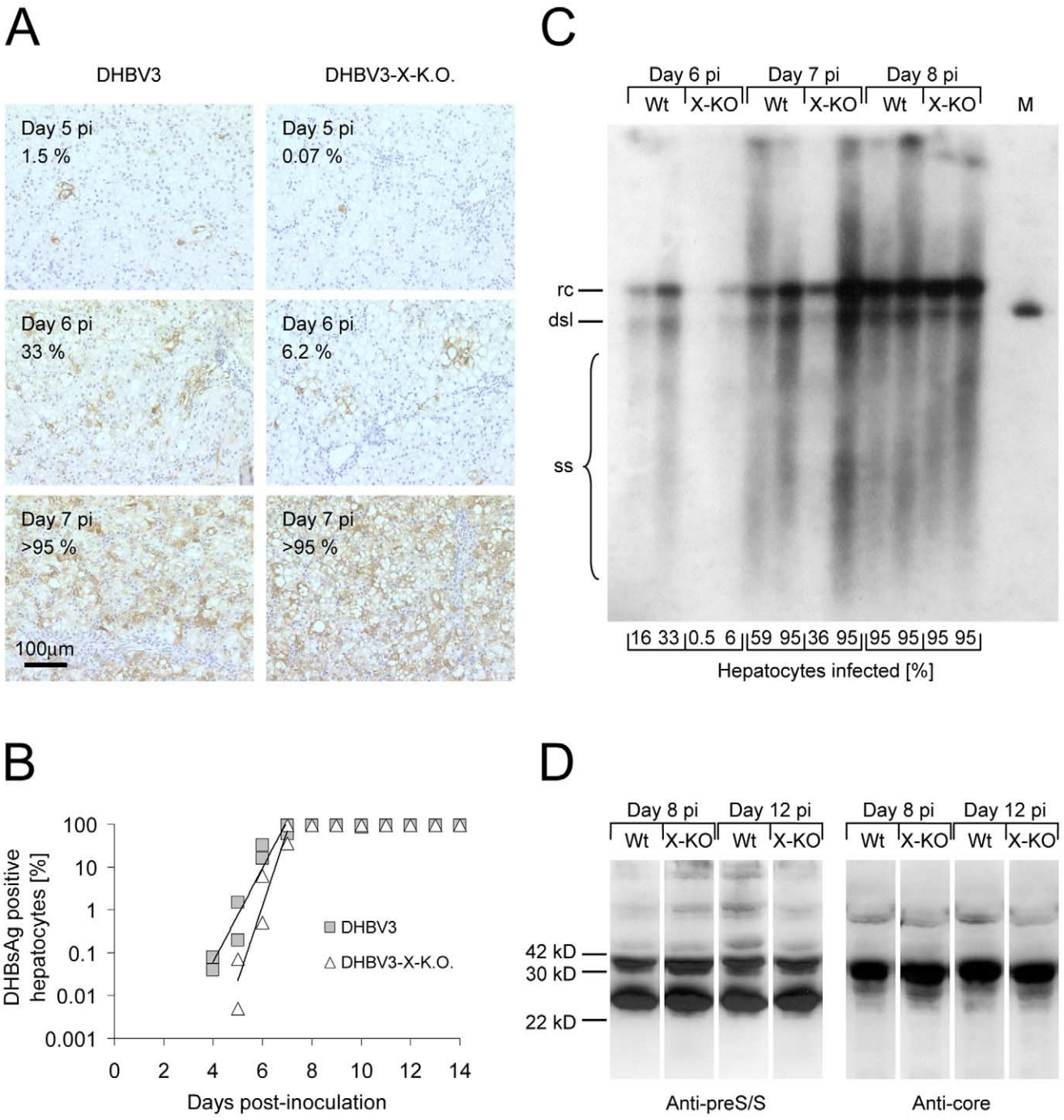

Fig. 2. Spread of virus in the liver. One- to 2-day-old DHBV-negative ducks were inoculated iv with 1500 ID_{50} of DHBV3 and DHBV3-X-K.O. Each day from days 3 to 14 p.i., two ducks from each group were autopsied and analyzed. (A) Liver sections collected on days 5–7 p.i. during the exponential phase of spread were stained for DHBsAg using 1H.1 monoclonal antibodies. Cell counts were performed as described under Materials and methods and resulting percentages of DHBsAg-positive hepatocytes were as indicated. The one duck of the pair analyzed exhibiting the higher percentage of infected cells is shown for each strain and each day. (B) Detection in DHBsAg-positive hepatocytes from days 4–14 p.i. Each symbol represents the percentage of infected hepatocytes in an individual duck. The lines shown are regression curves of data from the exponential phase of spread of DHBV3 and DHBV3-X-K.O., i.e., from days 4–7 p.i. (C) Southern blot hybridization of replicative intermediates. Seven micrograms of total DNA isolated from liver tissue from two ducks per strain per day was loaded into each lane. Percentages of infected hepatocytes for each duck are indicated for comparison. M, marker, 40 pg of linear DHBV DNA; rc, relaxed circular, dsl, double-stranded linear, and ss, single-stranded forms of viral DHBV DNA. (D) Western blot analysis. One hundred micrograms of total protein was loaded into each lane of a 12% polyacrylamide gel and after blotting DHBsAg and DHBcAg were detected using the 1H.1 monoclonal antibodies (specific to the PreS region of PreS/S) and rabbit serum containing antirecombinant DHBcAg antibodies, respectively. Wt, DHBV3 wild-type; X-KO, DHBV3-X-K.O.

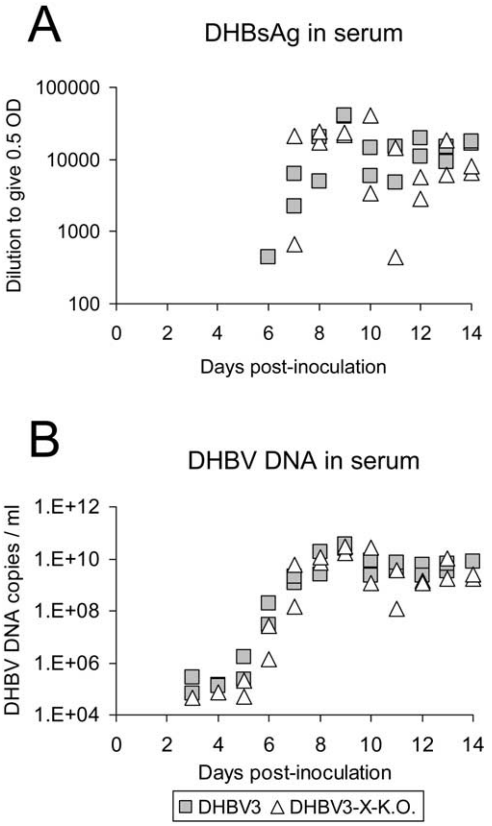

Fig. 3. DHBsAg and DNA in serum during spread of virus in the liver. (A) DHBsAg was detected and quantitated by ELISA. Each symbol represents the DHBsAg serum content of one duck determined by end-point titration as described under Materials and methods. DHBsAg was first detected at day 6 p.i. Serum DHBsAg levels in all ducks peaked at around day 9 p.i. and then declined by 0.5–1 log. (B) Total DNA was extracted from serum samples and DHBV DNA was quantitated using LightCycler FastStart DNA Master SYBR Green I. Each symbol represents the DHBV DNA content of one duck. DHBV DNA was first detected at day 3 p.i., peaked at day 9 p.i., and then declined by 0.5–1 log. The detection limit was equivalent to 1.1×10^4 DNA copies/ml.

with the highest dose, i.e., 10^8 ID_{50}, showed persistent infection, as expected from earlier results with the Aus-DHBV strain (Table 2) (Jilbert et al., 1998). Lower doses of 10^4 and 10^6 ID_{50} induced persistence only in 6/16 of the DHBV3-infected ducks and 4/16 of the DHBV3-X-K.O.-infected ducks, again showing no significant difference between the two strains.

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT) levels in serum from DHBV3- and DHBV3-X-K.O.-inoculated ducks analyzed on days 18 and 39 p.i. were mostly within normal ranges (Foster et al., 2003) and revealed no differences between the two strains (data not shown). Finally, in those ducks that developed persistent infection with the DHBV3 or DHBV3-X-K.O. strains with DHBsAg expression in >95% hepatocytes, the levels of expression of DHBsAg and DHBcAg in the liver were similar when measured by Western blot (data not shown).

Possible selection of X-expression revertant virus

It was possible that impaired fitness of the DHBV3-X-K.O. strain in vivo may not have been detected if rapid reversion to wt virus occurred soon after in vivo infection. However, serum samples from a total of 13 ducks inoculated with DHBV3-X-K.O. collected on days 10, 11, 13, 14, 35, 46, and 63 p.i. and the serum stock 1306 showed no reversion to wt when monitored by sequencing the X ORF of DHBV DNA amplified by PCR and using the amplification refractory mutation detection system (Newton et al., 1989) noreversion to wt DHBV3 was detected at a ratio of 1:1000 (data not shown).

Discussion

With mammalian hepadnaviruses, functional X protein plays an important though as yet undefined role in the full process of in vivo infection. DHBV strains contain an X ORF without an AUG start codon, but an X-like protein has recently been identified in DHBV-infected liver, and DHBx is expressed in vitro by wt but not DHBV3-X-K.O. Using in vitro assays, transactivation and apoptosis were shown to be markedly reduced when expression of DHBx was prevented by introducing a stop codon into the X-like ORF (Chang et al., 2001; Schuster et al., 2002).

In this study we have used the same DHBV DNA constructs as in the above work and a number of sensitive measures of in vivo fitness to examine whether a functional DHBx protein contributes to the in vivo infection process. The ultimate aim was to clarify the mechanism of any observed effect. We used the criteria of (i) quantitation of serum DHBV DNA, proteins, and infectivity in infected ducks with stable viraemia; (ii) the comparison of rate of spread of infection in liver and extrahepatic sites after low-dose inoculation; and (iii) the comparison of ability to produce transient or persistent infection under balanced age/dose conditions designed to detect small differences between the strains. No significant difference was seen between wt and DHBV3-X-K.O. strains by any of these measures. Furthermore, unlike the situation described with WHV (Zhang et al., 2001), the DHBV3-X-K.O. strain did not revert to wt as examined by direct sequencing of amplified viral DNA in any of the ducks tested, again providing

Table 2
The determination of viraemia in ducks from the age/dose experiment

Dose	DHBV3 wt	DHBV3-X-K.O.
10^4 ID_{50}	2/8[a]	3/8
10^6 ID_{50}	4/8	1/8
10^8 ID_{50}	8/8	8/8
Total	14/24	12/24

[a] Number of persistently infected ducks/total number of ducks inoculated.

no evidence for enhanced capacity of the wt virus to maintain infection in vivo.

Neither our current nor previous studies exclude expression of very low amounts of DHBx-like proteins from the DHBV3-X-K.O. genome that might be produced by translational read-through of the introduced stop codon or by translational initiation downstream of the stop codon at a nonconventional start codon. In addition, reversion of a minor fraction of DHBV3-X-K.O. to wt cannot be excluded by direct sequencing of amplified DNA or other methods. Both possibilities may provide sufficient DHBx for its in vitro and in vivo function. Furthermore, the possibility remains that X protein may play other role(s) in vivo that might not have been revealed in the above experiments, for example, during infection of adult ducks, in survival under natural conditions of transmission in ovo, or in host range specificity. The conservation of the X-like ORF across all the avihepadnaviruses suggests that some selection pressure may be operating in favor of X retention. On the other hand, the loss of the AUG in the X ORF of DHBV may indicate that, specifically with DHBV, this process may not be critical for virus survival.

Interestingly, recent in vitro experiments with HBV showed similar results to ours using HBV constructs in which HBx was knocked out (HBx-K.O.) and/or in which the phosphorylation sites in the PreS2 part of the large surface protein (LHBs) were mutated (LHBs-def.). These experiments showed that in the supernatant of HepG2 cells transfected with HBx-K.O. or LHBs-def. constructs, no significant differences in levels of viral DNA, HBsAg, or HBeAg were observed compared to wt transfected cells (Stockl et al., 2003). However, using a double-mutant HBx-K.O. combined with LHBs-def., a complete loss of HBsAg and HBeAg secretion was observed as well as dramatically diminished levels of viral DNA. HBV expression could be rescued by adding a fusion protein composed of a cell-permeable translocation motif (TLM) fused to either HBx or LHBs (Hafner et al., 2003). These results show that either HBx or functionally active LHBs are essential and sufficient for HBV gene expression. It would be interesting to determine if DHBV strains with mutations in both DHBx and DHBV PreS/S show a similar phenotype.

Materials and methods

Animals

One- to 2-day-old DHBV-negative Pekin–Aylesbury ducks (Anas domesticus platyrhyncos) were obtained from commercial duck hatcheries and held in the animal house facilities of the Institute of Medical and Veterinary Science (IMVS), Adelaide. All animal handling procedures were approved by both the IMVS and the University of Adelaide Animal Ethics Committees and followed the guidelines of the NHMRC.

Plasmids

The plasmid DNA used contained head-to-tail dimers of the genomes of DHBV3 (pDHBV3) or DHBV3-X-knock out (pDHBV3-X-K.O.) (Chang et al., 2001). The pDHBV3-X-K.O. carried a G-to-A change at nucleotide position 2371 of the DHBV3 strain, converting the codon for tryptophan (TGG) at amino acid position 28 in the X-like ORF into a stop codon (TAG). This change did not affect the overlapping ORF of the polymerase gene. Plasmid stocks were generated using commercial kits and the mutation in pDHBV3-X-K.O. was verified by direct sequencing of plasmid DNA.

DNA inoculation and DHBV infection

For the generation of viral stocks eight 1- to 2-day-old DHBV-negative ducks were inoculated ih (1305, 1308), iv (1304, 1307), or using combined routes ih and iv (1306, 1309, 1310, 1311) with 100 μg of pDHBV3 (1304, 1305, 1309, 1310, 1311) or pDHBV3-X-K.O. (1306, 1307, 1308) diluted in saline. The DHBV DNA and DHBsAg content of subsequent serum samples was determined by quantitative PCR and quantitative DHBsAg ELISA. For the determination of the ID_{50} of viral stocks, six groups of three 1- to 2-day-old DHBV-negative ducklings were inoculated iv with serial dilutions of the stocks of DHBV3 or DHBV3-X-K.O. analyzed above, using normal duck serum (NDS) as the diluent and containing the equivalent of 10,000, 1000, 100, 10, 1, and 0.1 viral genomes per inoculum dose. To analyze the spread of infection in the liver, two groups of 24 1- to 2-day-old DHBV-negative ducks were inoculated iv with 1500 ID_{50} of either DHBV3 or DHBV3-X-K.O., and each day from days 3 to 14 p.i., two ducks from each group were sacrificed and blood and tissue from liver, spleen, pancreas, and kidney were collected. Histological analysis and detection of DHBsAg were performed as described below. For the age/dose-related outcome, a total of six groups of eight 14-day-old DHBV-negative ducks were inoculated iv with 10^4, 10^6, or 10^8 ID_{50} of either DHBV3 or DHBV3-X-K.O., and the outcome of infection was monitored by detection of DHBsAg and DHBV DNA in serum.

Serological assays

To detect DHBsAg, ELISA plates (Costar 3590) were coated with a 1:100 dilution of test serum and blocked with 5% skim milk in PBS/0.05% Tween (Jilbert et al., 1996). DHBsAg was detected using 1H.1 monoclonal anti-DHBV preS antibodies (Pugh et al., 1995) at a dilution of 1:5000 followed by HRP-conjugated sheep anti-mouse IgG $F(ab')_2$ (Amersham, 1:4000) with the peroxidase substrate *o*-phenylene diamine (OPD; Sigma) and plates were read at 490 nm. The DHBsAg content of each sample was determined by end-point titration of serum samples using a pool of serum from congenitally DHBV-infected ducks (containing

50 μg/ml DHBsAg) (Jilbert et al., 1996) as a standard. NDS was added to the standard at the same dilution as the samples being tested, i.e., for samples diluted 1:1000, standards were diluted twofold from 1:500 to 1:256,000 and NDS diluted 1:1000 was added to each of the standards. For the analysis of liver enzymes in serum, samples were tested for levels of AST, ALT, and GGT using an automatic analyzer in the Diagnostic Services Laboratories of the IMVS. The normal range for the level of each enzyme in NDS was determined to be 15.9 ± 5.9 U/L for AST, 26.6 ± 7.7 U/L for ALT, and 2.3 ± 1.2 U/L for GGT (Foster et al., 2003).

Detection and quantification of serum DHBV DNA by PCR and sequencing of PCR-amplified fragments

DHBV DNA was isolated from serum samples using High Pure Viral Nucleic Acid Kit (Roche) that included proteinase K treatment in the first step. The DHBV DNA content was determined by quantitative PCR (Roche LightCycler) using LightCycler FastStart DNA Master SYBR Green I (Roche). A plasmid containing a head-to-tail dimer of AusDHBV DNA was used as a standard (Triyatni et al., 2001). Samples consisted of 1 μl of duck serum extract diluted 1:100 (equivalent to 0.04 μl of duck serum), 2 μl of FastStart DNA Master SYBR Green I, 2.4 μl $MgCl_2$ (final concentration 4 mM), 0.5 μl of 10 μM primers (final concentration for each primer 0.25 μM) in a total of 20 μl. Primers used for quantitative PCR were located in conserved regions of the surface (S) ORF of AusDHBV: P3 (5′ AGCTGGCCTAATCGGATTAC3′) was located at nt 1316–1335 and P4 (5′TGTCCGTCAGATACAGCAAG3′) was located at nt 1565–1584. For sequencing, serum DHBV DNA was precipitated by polyethylene glycol, and the X ORF region was amplified using primers P1 (5′CAACACATGGCGCAATATCC3′), located at nt 2175–2194, and P2 (5′TGTGTAGTCTGCCAGAAGTC3′) located at nt 2821–2840. For the sequencing reaction, the BigDye Terminator v3.0 cycle sequencing kit (Applied Biosystems) was used according to the manufacturer's protocol with primer P1 as the sequencing primer. Analysis was performed on an ABI Prism 3700.

Tissue analysis

For Western blot analysis, duck liver was homogenized in PBS containing 0.5% NP-40 to give a 10% homogenate. The total protein content of the homogenates was determined using a commercial protein determination assay (Bio-Rad). Samples containing 100 μg of total protein were mixed with sodium dodecyl sulphate (SDS) loading buffer containing β-mercaptoethanol, boiled, and separated by SDS–polyacrylamide gel electrophoresis. Proteins were blotted onto Hybond ECL nitrocellulose membranes (Amersham) that were blocked overnight with 5% skim milk. DHBsAg was detected using 1H.1 (1:5000) and HRP-conjugated sheep anti-mouse Ig $F(ab')_2$ (1:4000). DHBcAg was detected using rabbit anti-recombinant DHBcAg antibodies (1:1000) (Jilbert et al., 1992), followed by a goat anti-rabbit serum containing HRP (1:4000; KPL, Gaithersburg, MD, USA) and ECL+plus (Amersham) and membranes were exposed on Kodak X-OMAT AR. For Southern blot hybridisation, total DNA was isolated from 25 mg liver tissue samples using a DNeasy tissue kit (Qiagen) that included proteinase K treatment in the first step. Seven-microgram samples of total DNA were loaded into each lane of a 1.2% agarose gel, and blotting and detection using a ^{32}P-labeled probe was performed as described previously (Jilbert et al., 1992). Immunohistological analysis was performed as follows: DHBsAg was detected in sections of ethanol:acetic acid (EAA, 3:1) fixed, wax-embedded liver, spleen, pancreas, and kidney tissue, after blocking of endogenous peroxidase with H_2O_2, by standard immunoperoxidase staining (Jilbert et al., 1996) using 1H.1 monoclonal antibodies followed by HRP-conjugated sheep anti-mouse Ig $F(ab')_2$ (Amersham) and diaminobenzidine (DAB; Sigma). Sections were counterstained with hematoxylin, dehydrated, and mounted in Depex. Nuclei of hepatocytes staining positive for DHBsAg were counted with the aid of an eyepiece graticule in 3–100 (most commonly 10) 250 × 250 μm grid fields and expressed as a percentage of the average total hepatocyte nuclei counted in three representative grid fields for each duck. Histological analysis was performed using sections of formalin-fixed wax-embedded tissue stained by haematoxylin and eosin (H&E).

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