

Replication of hepatitis B virus in primary duck hepatocytes transfected with linear viral DNA

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Abstract

AIM: To explore the expression and replication of hepatitis B virus (HBV) DNA in primary duck hepatocytes (PDHs).

METHODS: Complete HBV genome was transfected into PDHs by electroporation (transfected group, 1.19×10^{12} copies of linear HBV DNA/ 1×10^7 PDHs). After 1-5 d of transfection, HBsAg and HBeAg in the supernatant and lysate of PDHs were measured with the IMX System. Meanwhile, replicative intermediates of HBV DNA were analyzed by Southern blotting and Dot blotting. PDHs electroporated were used as control group.

RESULTS: HBsAg in the hepatocyte lysates of transfected group was 15.24 (1 d), 14.55 (3 d) and 5.13 (5 d; P/N values, positive ≥ 2.1) respectively. HBeAg was negative (< 2.1). Both HBsAg and HBeAg were negative in the supernatant of transfected group. Dot blotting revealed that HBV DNA was strongly positive in the transfected group and negative in the control group. Southern blot analysis of intracellular total DNA indicated that there were relaxed circular (rc DNA), covalently closed circular (ccc DNA), and single-stranded (ss DNA) HBV DNA replicative intermediates in the transfected group, there was no integrated HBV DNA in the cellular genome. These parameters were negative in control group.

CONCLUSION: Expression and replication of HBV genes can occur in hepatocytes from non-mammalian species. HBV replication has no critical species-specificity, and yet hepatic-specific regulating factors in hepatocytes may be essential for viral replication.

INTRODUCTION

Hepatitis B is one of the fatal diseases all over the world, and its pathogenesis is still unclear and its therapy appears difficult too. In recent years, reports have revealed that hepatocytes were not only the target cells infected with hepatitis B virus (HBV) and destructed by activated cytotoxic T lymphocytes, but also the immune modulating cells activated by effector cells, and even take part in the process of HBV non-cytolytic clearance^[1,2]. In order to study interactions between HBV and hepatocytes, we established an *in vitro* model of primary culture hepatocytes from heterologous species transfected with linear HBV DNA. Since whether naked HBV DNA can replicate or express protein in non-human hepatocytes, and whether its replication process is similar to that in normal specific host are not reported, we transfected linear HBV DNA into primary duck hepatocytes (PDHs) to investigate the host-specific regulating role in the replication of HBV in hepatocytes from non-mammalian species.

MATERIALS AND METHODS

Isolation and culture of primary duck hepatocytes

Duck hepatitis B virus (DHBV) DNA and DHBsAg in duck serum were negative tested by Dot blot analysis and ELISA respectively. Hepatocytes were harvested from Chongqing ducks using an *in situ* collagenase perfusion technique^[3]. The ducks were injected into the peritoneal cavity pentobarbital sodium (30 mg/kg body weight) and heparin (100 IU/kg body weight), the abdomen of the animals was opened and the portal vein was exposed and cannulated. Then the liver was *in situ* perfused at 37 °C with calcium-free, Hanks' balanced salt solution (HBSS) for

10 min, then with 0.2 g/L collagenase type I in calcium-presenting HBSS for 15 min. The liver was removed and the cells were combed gently in tissue culture medium. Hepatocytes were centrifugated, washed, and separated from non-parenchymal cells by differential centrifugation at 50 g. Viability of hepatocytes detected by trypan blue exclusion (TBE) was about 90%, and the cells were counted.

Freshly isolated hepatocytes were transfected with linear HBV DNA, and then inoculated at a density of 3×10^6 cells per 25 cm² culture flask. Culture medium was composed of RPMI 1640 with insulin (100 IU/L), penicillin, streptomycin and 100 mL/L fetal bovine serum. Cultures were incubated at 37 °C in a humidified atmosphere containing 50 mL/L CO₂ and media were renewed every two days.

Extraction of linear HBV DNA

Plasmid pEcoB 6 containing two *EcoRI* copies of HBV genome in a head-to-tail arrangement was used to extract the linear HBV DNA. Extraction was carried out as previously described^[4]. Briefly, pEcoB 6 was digested by *EcoRI* at 37 °C for 2 h, then fractionated by electrophoresis on 1% agarose gel. The complete HBV DNA 3.2 kb fragment was extracted and quantified for transfection.

Transfection procedures

Transfection was performed as previously described^[5], electroporation conditions were optimized by Yao *et al.* In brief, 4 μg linear HBV DNA was added to 1×10^7 hepatocytes and electroporated for about 29 ms at 220 V and 950 μF of capacitance. After gene transfer, the cells were inoculated and cultured for 5 d. The electroporated hepatocytes were used as control.

Test for HBV-specific proteins

After 1-5 d of transfection, HBsAg and HBeAg in the supernatant and lysate of hepatocytes were identified using radioimmunoassay kits (Abbott Laboratories) according to the manufacturer's instructions. The HBsAg signal/noise (P/N ratio) >2.1 was considered positive.

DNA extraction from hepatocytes and HBV DNA replication analysis

Intracellular total DNA was extracted 2 d after transfection. Southern blotting was used to analyze replicative intermediates of HBV DNA. Dot blotting was used to test the total HBV DNA in hepatocytes from d 1 to 5 after transfection. All procedures were performed as previously described^[4].

RESULTS

Viral antigen production in transfected duck hepatocytes

Production of HBsAg and HBeAg was measured in culture supernatant and cell lysate was collected daily from transfected hepatocytes group. HBsAg in the lysate of transfected hepatocytes increased during the first 3 d following transfection, with HBsAg P/N value being around 15 (Table 1). HBsAg was negative in all culture supernatants of transfected hepatocytes. Both lysate and supernatant were negative for HBeAg. Both HBsAg and HBeAg in control group were negative.

Table 1 HBsAg values (P/N) in primary duck hepatocyte lysates

Time after transfection	1 d	3 d	5 d
Transfected group	15.24	14.55	5.13
Control group	1.01	0.93	1.38

DNA analysis of intracellular total DNA

Dot blotting revealed that total amount of HBV DNA in transfected hepatocytes was strongly positive from d 1 to 5 following transfection (Figure 1). Southern blot analysis of intracellular total DNA indicated that, there were relaxed circular (rc DNA), covalently closed circular (ccc DNA) and single-stranded (ss DNA) HBV DNA replicative intermediates in the transfected hepatocytes (Figure 2). There was no integrated viral DNA in the cellular genome.

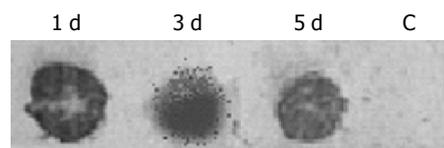


Figure 1 Dot blotting test of HBV DNA in transfection group. C: control group.

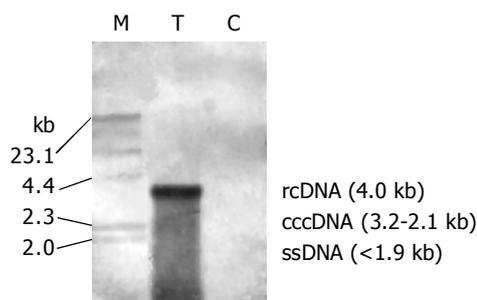


Figure 2 Southern blotting analysis of HBV DNA replication in transfected hepatocytes. M: marker of standard molecular weight; T: transfection group; C: control group.

DISCUSSION

DHBV *in vivo* and *in vitro* models have been used for the study of HBV infection and the evaluation of new anti-HBV strategies^[6-10]. However, until now, there is no report on the process of viral infection, the level of DNA replication and protein expression and the mechanisms of hepatitis B virus in hepatocytes from heterologous species. Whether naked HBV DNA can replicate or express protein in nonhuman hepatocytes, and whether its replication process is similar to that in normal specific host are still unclear. We transfected linear HBV DNA into non-mammalian hepatocytes, PDH to investigate the host-specific regulating role in the replication of HBV.

Our results indicate that naked DNA of HBV can effectively replicate in PDHs and its replicative intermediates include relaxed circular DNA, covalently closed circular DNA, single-stranded DNA, and non integrated viral DNA

in the cellular genome. The pattern of DNA replication of HBV is similar to that in normal permissive human cells and in liver of chimpanzee acutely infected with HBV^[11], which is the same as that of DHBV replication in liver of chronically infected duck^[12], and is further supported by the results of PDHs acutely or chronically infected with DHBV^[3,13]. Thus complete HBV genome effectively transfected into primary hepatocytes can efficiently replicate.

Our results also show that HBV can effectively express proteins in primary duck hepatocytes, such as HBsAg, with its P/N value peak being around 15.0 1-5 d after transfection. In our study, HBsAg was found only in the lysate of duck hepatocytes, but not in the supernatant of cultured hepatocytes, which can be explained that HBsAg is insufficient to excrete in outer media of hepatocytes, and unbalance between large, mediate and small proteins of HBsAg might also affect its secretion. Furthermore, it needs further study. No HBeAg was measured in the lysate or supernatant of duck hepatocytes, which might be related to two reasons. The first, is that in duck hepatocytes there is no sufficient translation activators essential for effective activation of HBV core gene promoter. Yu and Mertz^[14,15], found that HBV pre-C RNAs and pre-genome RNAs are separately regulated by two activators. The other reason is that shortly after expression, HBeAg is digested by lysozymes in duck hepatocyte plasma. The real causes need further study.

In conclusion, expression and replication of HBV genes can occur in hepatocytes from non-mammalian species, which strongly supports the idea that replication of HBV has no critical species-specificity, and hepatic-specific regulating factors in hepatocytes are essential for viral replication. Further more, the differences in inner environments of hepatocytes from different species affect and even determine the expressions of HBV genes and proteins.

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