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Adeno-associated virus Rep78 protein inhibits Hepatitis B virus replication through regulation of the HBV core promoter

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ABSTRACT

Rep78, the *rep* gene product of adeno-associated virus (AAV), has been shown to inhibit the replication of several DNA viruses. This study investigated the effects of Rep78 on replication of Hepatitis B virus (HBV) and possible mechanisms of inhibition. We have shown that HBV DNA replication and secretion of HBsAg and HBeAg in HepG2 2.2.15 cells were inhibited by Rep78. We have also demonstrated, using *in vitro* transcription and luciferase assay, that Rep78 binds to the HBV core promoter (HBV CP) and inhibits HBV CP activity. Furthermore, after Rep78 and HBV core protein expression plasmids were co-transfected into HepG2 cells, the expression of HBV core protein was inhibited significantly. These results suggest that Rep78 can inhibit the replication of HBV, correlating strongly with suppression of HBV CP activity.

Introduction

Adeno-associated virus is known to have inhibitory effects on the replication of several DNA viruses [1,2]. This property has been attributed to AAV's nonstructural major regulatory protein, Rep78 [3]. Previous studies have shown that Rep78 was able to interfere with the transcription of many viral promoters, including those of adenovirus, human papillomavirus, herpes simplex virus and human immunodeficiency virus [4–6].

In China, chronic HBV infection is still the major cause of liver cirrhosis and hepatocellular carcinoma. We investigated the possible effects of Rep78 on HBV. Our results show that Rep78 can inhibit the replication of HBV in HepG2 2.2.15 cells, a broadly used cell line to study HBV replication. Additionally, the release of both HBsAg and HBeAg into supernatants was diminished. The HBV CP plays an important role in HBV replication [7,8] and here we demonstrated that AAV Rep78 binds to the HBV CP specifically *in vitro* and inhibits transcription and expression of the CP. These results suggest that the inhibition of HBV DNA replication by Rep78 could be correlated with the regulatory control of Rep78 on the HBV CP.

Materials and methods

Transient transfection of HepG2 2.2.15 cells with Rep78 expression plasmid and intracellular HBV DNA analysis. To generate the Rep78 expression plasmid, pcDNA-Rep78, the coding regions for Rep78 were amplified by PCR. The primers used for amplification of Rep78 were 5′-ATGATATCCATGCCGGGGTTTTACGAGA-3′ and 5′-ATCTCGAGTCATTTATTGTTCAAAGA-3′. The PCR product was digested with EcoRV and XhoI and inserted into a pcDNA4/zeocin plasmid cut with EcoRV and XhoI. HepG2 2.2.15 cells were transfected with 5.0 μg pcDNA-Rep78 plasmid. After 3 days, total DNA from the transfected HepG2 2.2.15 cells was isolated, quantified and normalized. Southern blotting was performed using a digoxin-labeled HBV DNA probe.

Detection of HBsAg and HBeAg in the culture supernatants. HepG2 2.2.15 cells were transfected with 5.0 μg pcDNA-Rep78 plasmid. Culture supernatants from HepG2 2.2.15 cells were sampled at 3 and 6 days post-transfection. HBsAg and HBeAg were detected by electrochemiluminescence immunoassay using an Architect i2000 analyzer (Abbott Diagnostics, Wiesbaden, Germany) according to the manufacturer's instructions. The concentrations of HBsAg and HBeAg were expressed in IU/mL and S/CO, respectively.

Purification of maltose-binding protein (MBP)-Rep78. AAV Rep78 was expressed in *Escherichia coli* as an MBP fusion protein encoded by the pMAL-Rep78 plasmid [9]. MBP-Rep78 exhibits all the known biochemical functions of wild-type Rep78. MBP-Rep78 was purified by affinity chromatography using amylose resin, according to the manufacturer's instructions (New England Biolabs, MA, USA). The purified fractions were examined by 10% SDS–PAGE, and Western blotting using an anti-MBP antibody.

Electrophoretic mobility shift assays (EMSAs). HBV CP DNA was prepared by performing PCR using the following primers: 5′-CAACGACCGACCTTGAGGCATA-3′ and 5′-TGGAGGCTTGAACAGTA

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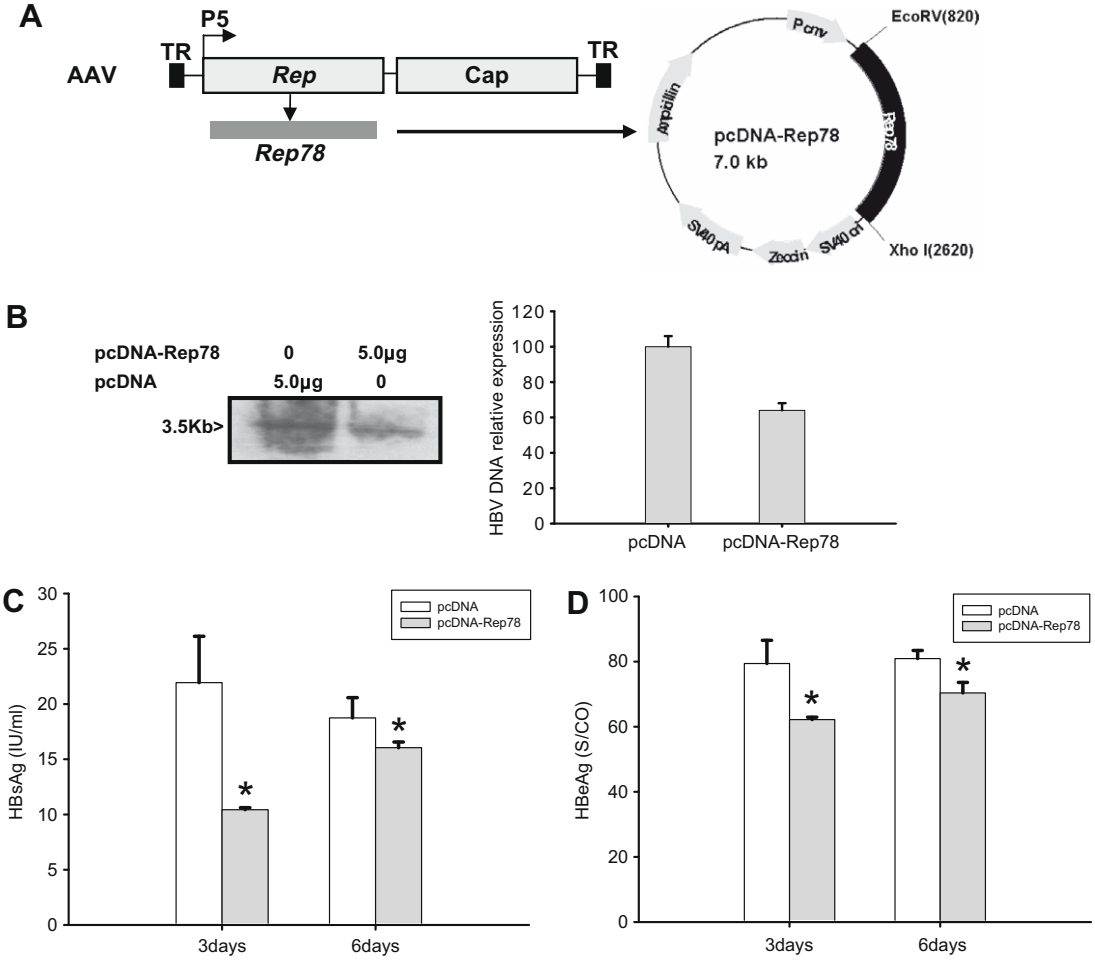

Fig. 1. Rep78 inhibits the replication of HBV and the secretion of HBsAg and HBeAg in HepG2.2.15 cells. (A). Schematic diagram of AAV genome and construction of the Rep78 expression plasmid (pcDNA-Rep78). (B). HepG2 2.2.15 cells were transfected with 5.0 μg pcDNA-Rep78 or pcDNA. At 3 days post-transfection, Southern blotting results showed inhibition of HBV replication. (C,D). HBsAg and HBeAg in culture supernatants were detected at 3 and 6 days post-transfection. Both HBsAg and HBeAg were significantly inhibited ($^{*}p < 0.05$).

GG-3′. Binding reactions were performed in 20 μL of binding buffer, containing 2.5% glycerol, 5 mM $MgCl_2$, 2 ng poly(dI-dC), 20 mM EDTA, 20 fmol digoxin-labeled CP DNA and MBP or MBP-Rep78 protein. For competitive experiments, a 10-fold molar excess of unlabeled CP DNA was incubated with MBP-Rep78 protein for 15 min at room temperature before adding the labeled CP DNA.

In vitro transcription reactions. Transcription reactions were performed using a HeLaScribe® Nuclear Extract *in vitro* Transcription System (Promega, WI, USA). A DNA fragment incorporating the CP and the full length of the C gene (HBV-C, 783 bp) was obtained by PCR. The primers used to amplify this HBV-C fragment were 5′-CAACGACCGACCTTGAGGCATA-3′ and 5′-GAGTCCAAGGGATACTAAC-3′. The 25 μL reaction mixture contained eight units of HeLa nuclear extract, 1× transcription buffer, 3 mM $MgCl_2$, 100 ng HBV-C DNA fragment, digoxin-labeled NTP and 100 ng MBP or MBP-Rep78 protein. The reactions were incubated at 30 °C for 60 min. Reaction products were phenol-chloroform extracted and ethanol precipitated. The RNA was dissolved in loading dye and separated on a 6% acrylamide/7 M urea/1× TBE gel.

Luciferase reporter gene assays. A luciferase reporter construct, pGL3-CP, was made by cloning the HBV CP DNA fragment into a pGL3-Basic vector (Promega, USA) between the MluI and XhoI sites. Approximately 500 ng of each vector, pGL3-CP and pcDNA-Rep78, were co-transfected into HepG2 cells using FuGENE® HD Transfection Reagent (Roche Diagnostics, IN, USA). A 20 μL aliquot of cell lysate was then assayed for luciferase activity. To correct for variations in transfection efficiencies among experiments, every dish of cells was transfected with pRL-TK (Promega, USA), an internal control vector containing a *Renilla* luciferase reporter gene. The luciferase activity of the pGL3-CP construct was normalized to *Renilla* luciferase and expressed as a fold-change of the activity achieved with the control vector, pRL-TK.

Construction of HBV core protein expression plasmid (pcDNA-HBV-C) and transfection of HepG2 cells. A fragment including the promoter and coding regions for the HBV core protein was generated by PCR amplification. The primers used were 5′-ATGATATCCCCAAGGTCTTACATAAGA-3′ and 5′-ATCTCGAGGAGTCCAAGGGATACTAAC-3′. The PCR product was digested with EcoRV and XhoI and inserted into a pcDNA4/zeocin plasmid cut with the same restriction enzymes. HepG2 cells were co-transfected with 0.5 μg pcDNA-HBV-C and 0.5 μg pcDNA-Rep78. Cells were harvested 72 h after transfection and the expression of HBV core protein and Rep78 detected by immunoblotting using antibodies to core protein (Abcam, Cambridge, UK) or Rep78 (Fitzgerald, MA, USA), respectively.

Statistical analysis. Statistical analysis was performed using the Student's *t*-test. A *p* value less than 0.05 was considered significant.