Biochemical and Biophysical Research Communications 385 (2009) 106–111

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Tianhui Liu [a], Min Cong [a], Ping Wang [a], Jidong Jia [a], Yong Liu [b], Paul L. Hermonat [b], Hong You [a,*]

[a] Liver Research Center, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, PR China
[b] Gene Therapy Center, Department of Internal Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

ARTICLE INFO

Article history:
Received 9 April 2009
Available online 3 May 2009

Keywords:
Hepatitis B virus
Core promoter
Rep78
Replication
Transcription

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

* Corresponding author. Address: Liver Research Center, Beijing Friendship Hospital, Capital Medical University, No. 95 YongAn Road, XuanWu District, Beijing 100050, China. Fax: +86 10 83165944.
E-mail address: youhong30@sina.com (H. You).

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doi:10.1016/j.bbrc.2009.04.132

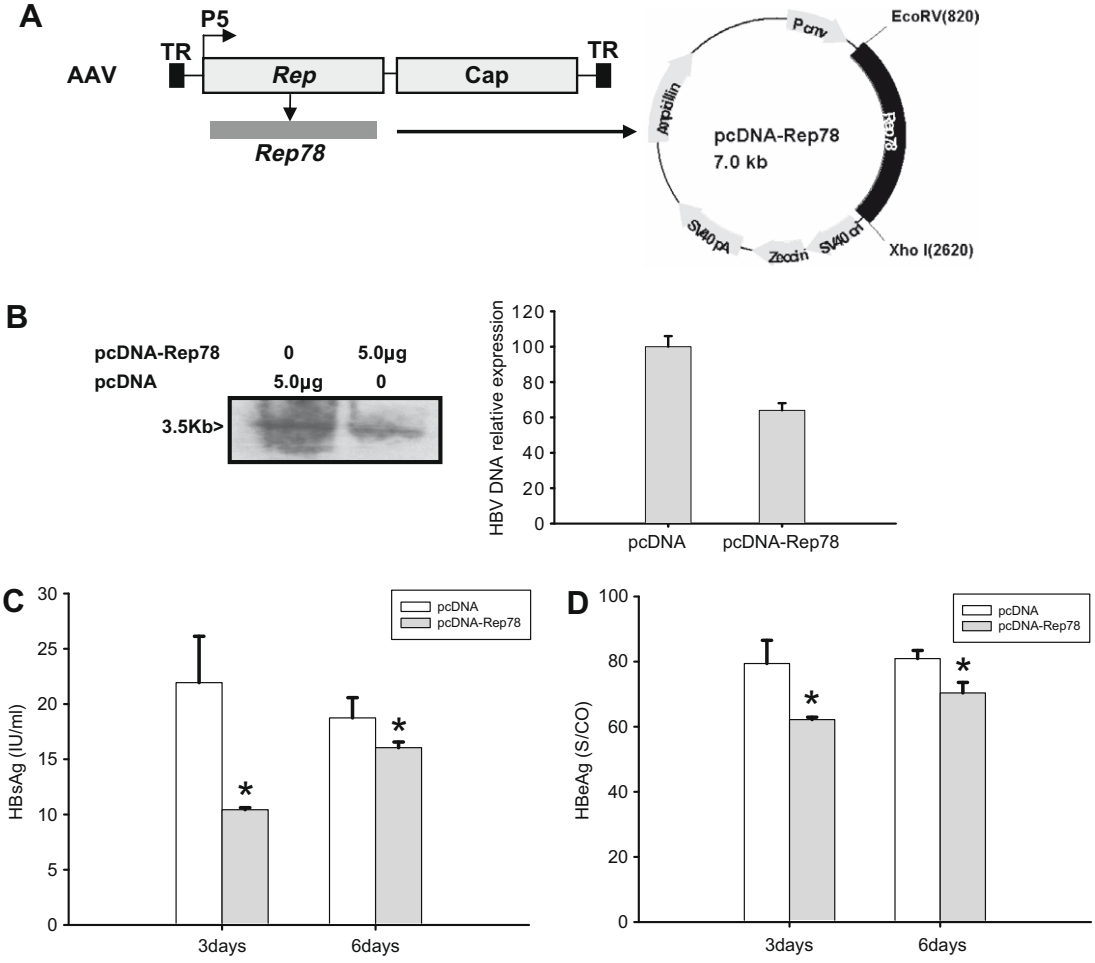

Fig. 1. Rep78 inhibits the replication of HBV and the secretion of HBsAg and HBeAg in HepG2.2.15 cells. (A). Schematic diagram of AAV genome and construction of the Rep78 expression plasmid (pcDNA-Rep78). (B). HepG2 2.2.15 cells were transfected with 5.0 μg pcDNA-Rep78 or pcDNA. At 3 days post-transfection, 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.

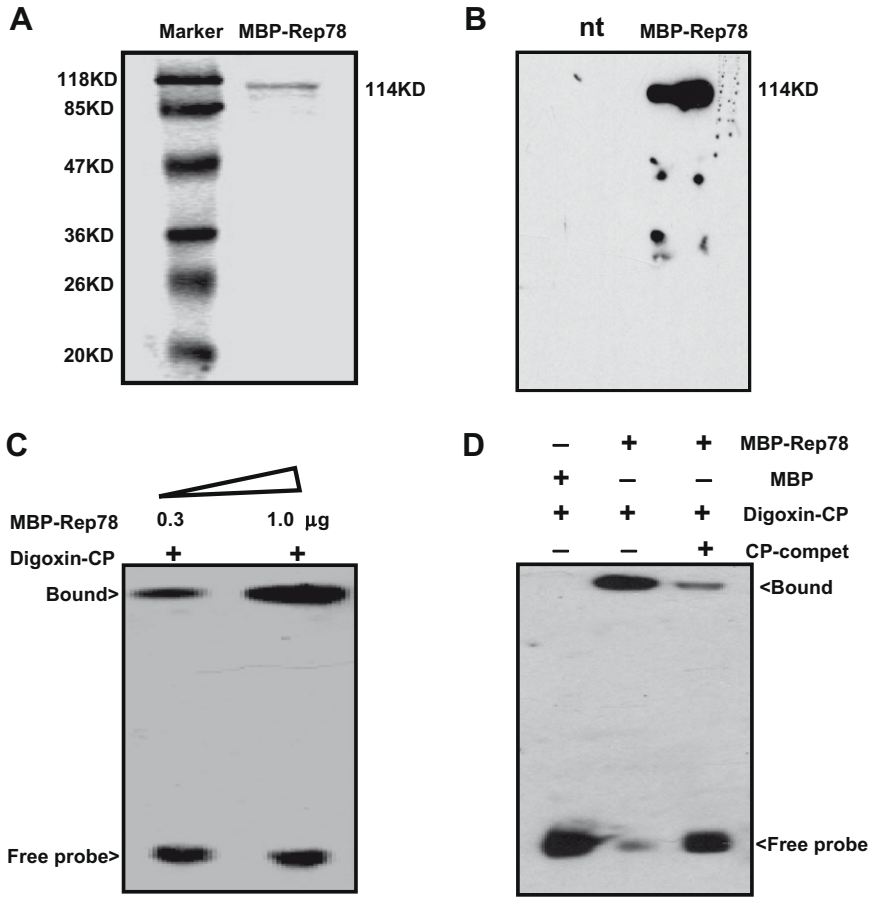

Fig. 2. Analysis of MBP-Rep78 and its specific binding to HBV CP demonstrated by EMSA. (A) Rep78 was expressed in *E. coli* as an MBP fusion protein encoded by pMAL-Rep78. 1.0 mg MBP-Rep78 protein was analyzed by 10% SDS–PAGE. The expected size of MBP-Rep78 protein was 114 kDa. (B) The purified MBP-Rep78 fusion protein was electroblotted and probed with a monoclonal MBP antibody. Protein extract from *E. coli* cells without IPTG induction was used as a negative control (nt). (C) Digoxin-labeled CP (Digoxin-CP) was incubated with MBP-Rep78, and the products were fractionated on a non-denaturing polyacrylamide gel. EMSA results showed that MBP-Rep78 bound to HBV CP in a dose-dependent manner. (D) Competitive EMSA experiments analyzed MBP-Rep78 binding to Digoxin-CP with competition from unlabeled CP (CP-compet). The competition with HBV CP was seen at a ratio of 1:10 labeled to unlabeled HBV CP DNA.

Results

Rep78 inhibits the replication of HBV DNA and the secretion of HBsAg and HBeAg in HepG2 2.2.15 cells

A Rep78 expression plasmid, pcDNA-Rep78, was constructed (Fig. 1A) and used to transfect HepG2 2.2.15 cells. Cells were transfected with 5.0 μg of either pcDNA-Rep78 or the control vector, pcDNA. HBV DNA was extracted and analyzed by Southern blotting 72 h post-transfection. As shown in Fig. 1B, Rep78 inhibited the replication of HBV. Culture supernatants from HepG2 2.2.15 cells were also collected and assayed for the presence of the HBV antigens, HBsAg and HBeAg, at days three and six post-transfection. It can be seen that the release of both HBsAg and HBeAg into the culture supernatants was significantly inhibited (Fig. 1C and D) ($p < 0.05$).

Rep78 specifically binds to HBV CP

EMSAs were performed with purified MBP-Rep78 (Fig. 2A and B) to detect any interactions between Rep78 and HBV CP. Fig. 2C shows MBP-Rep78 has shifted the HBV CP DNA in a dose-dependent manner. To determine if the binding was specific for HBV CP, unlabeled HBV CP DNA was used as a competitor in the binding assay. Competition with HBV CP was seen at a ratio of 1:10 labeled to unlabeled HBV CP DNA, indicative of specific binding of Rep78 to HBV CP (Fig. 2D).

Rep78 inhibits the activity of HBV CP

To further investigate the biological significance of the interaction between Rep78 and the CP, *in vitro* transcription assays were used. Representative experiments are shown in Fig. 3A, indicating that MBP-Rep78 proteins exerted significant inhibition on transcription of the HBV C gene, whereas the addition of MBP proteins alone had no effect on the level of HBV C-RNA (783 nt.). A luciferase reporter assay was also performed to further verify the effect. A reporter plasmid (pGL3-CP) containing the HBV CP coupled to a firefly luciferase gene was constructed (Fig. 3B) and transfected into HepG2 cells in combination with pRL-TK and pcDNA-Rep78. Both firefly and *Renilla* luciferase activities were measured, and the results demonstrated that Rep78 inhibited CP activity (Fig. 3B) ($p < 0.05$).

Rep78 inhibits the expression of HBV core protein

After determining Rep78 specifically bound to the HBV CP and inhibited its activity, we investigated the effects of Rep78 on the expression of the HBV core protein. The vector pcDNA-HBV-C was constructed (Fig. 4A) and co-transfected with 0.5 μg pcDNA-Rep78 into HepG2 cells. It was clearly demonstrated that Rep78 could inhibit HBV core protein expression when extracted protein from the transfected cells was analyzed (Fig. 4B).

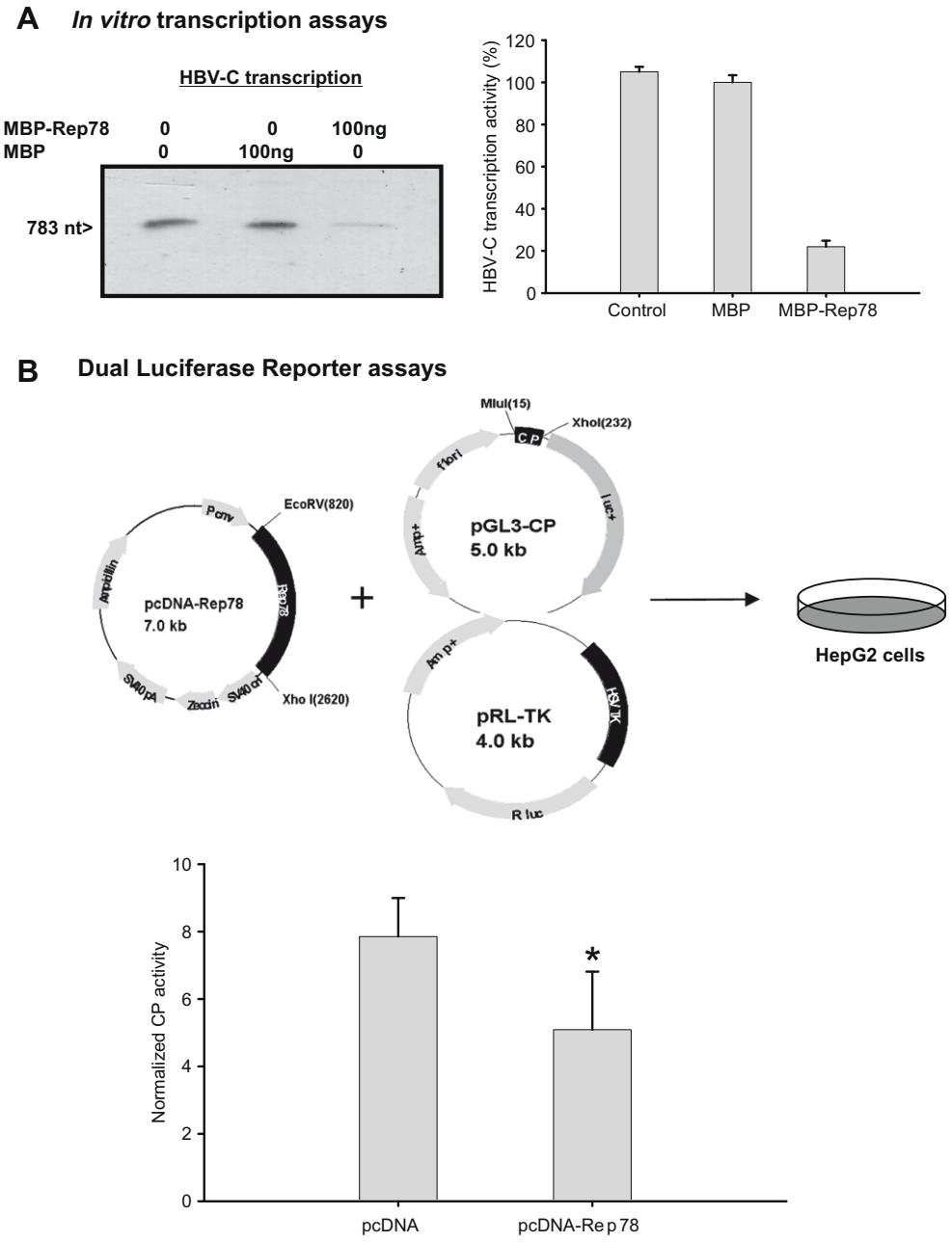

Fig. 3. Rep78 inhibits the transcription of HBV CP *in vitro*. (A). *In vitro* transcription assays were performed with HeLa nuclear extracts, the linearized HBV-C DNA and 100 ng MBP or MBP-Rep78, as described in materials and methods. The digoxin-labeled products were then analyzed by UREA–PAGE. The addition of MBP-Rep78 resulted in a significant inhibition of transcription of the HBV C DNA, whereas the addition of MBP proteins alone had no effect on the level of C RNA (783 nt.). (B). Construction of a CP luciferase reporter plasmid, pGL3-CP. HepG2 cells were transfected with 500 ng pGL3-CP, and combinations of 500 ng pRL-TK and 500 ng pcDNA-Rep78. Both firefly and *Renilla* luciferase activities were measured. pGL3-CP firefly luciferase activity was normalized to *Renilla* luciferase activity. Rep78 inhibited CP activity ($^*p < 0.05$). Data was compiled from four independent experiments in HepG2 cells.

Discussion

AAV Rep78 protein could inhibit several DNA viruses. We tried to investigate whether Rep78 could inhibit HBV replication. HepG2 2.2.15 cells were used in this study as they can stably replicate HBV DNA and express HBsAg and HBeAg, thereby making them a suitable cell model to screen the *in vitro* antiviral efficacy of Rep78. We have demonstrated that Rep78 protein is able to inhibit the replication of HBV DNA in HepG2 2.2.15 cells. Additionally, the secretion of HBsAg and HBeAg in the culture medium of HepG2 2.2.15 cells was significantly inhibited in the presence of Rep78.

Since Rep78 protein could bind to a variety of promoters in DNA viruses [4–6], it could act as a transcriptional regulator. Thus, we then tried to investigate if this inhibition of Rep78 on HBV correlated with binding between Rep78 and the HBV CP. In this study, we have shown that Rep78 binds to the HBV CP DNA and inhibits its activity. Furthermore, co-transfection of HBV core protein and Rep78 expression plasmids into HepG2 cells demonstrated that the expression of HBV core protein was inhibited significantly. These results suggest both transcription and gene expression from the HBV CP are inhibited by Rep78.

It has been reported that Rep78 has a general cytotoxic effect on host cells [10,11]. The observed inhibition in our study could

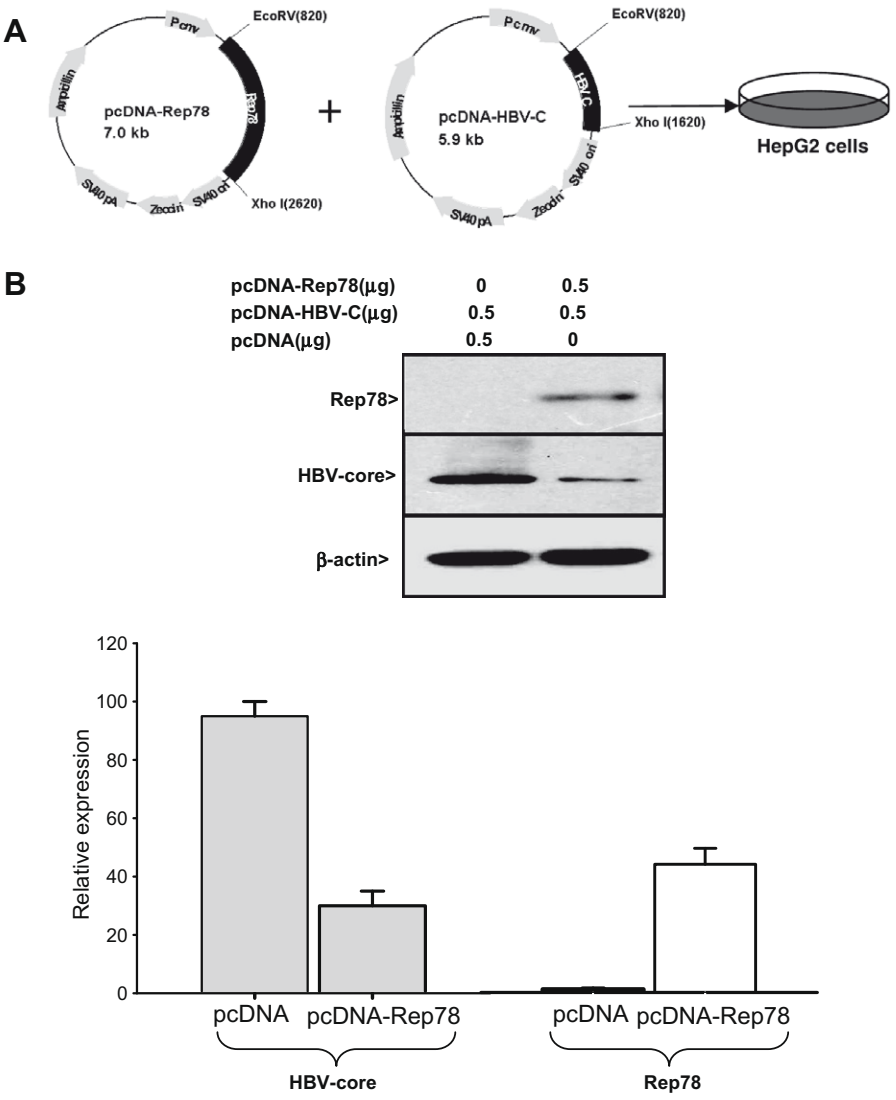

Fig. 4. The expression of HBV core protein was inhibited by Rep78. (A). Construction of HBV core protein expression plasmid, pcDNA-HBV-C. (B). HepG2 cells were transfected with 0.5 μg pcDNA-HBV-C and in combination with 0.5 μg pcDNA-Rep78. Rep78 protein and HBV core protein were detected by immunoblotting with the monoclonal Rep78 antibody and core protein antibody, respectively. Protein extracted from cells transfected with pcDNA plasmid without Rep78 coding sequences and pcDNA-HBV-C was analyzed as a control. The expression of HBV core protein was inhibited by Rep78.

therefore be due to the cytotoxic effect of Rep78. However, analysis of cell lines expressing Rep78 has shown that the presence and expression of Rep78 does not necessarily lead to cell death [12,13]. In our study, there is no significant change in the proliferation of cells after transient transfection with an appropriate concentration of pcDNA-Rep78 (less than 0.5 μg/5 × 10^4 cells) into HepG2 or HepG2 2.2.15 cells (data not shown).

Rep78 is a multifunctional protein that has a general effect on host cells [14]. The Rep78-CP interaction might not be the only event responsible for the inhibition of HBV replication. Protein–protein interactions involving specific protein sequences, or general transcription factors, might also be involved in the inhibition. Rep78 has been shown to bind to several cellular proteins, including transcription factors such as TBP, AP-1, Sp1 [15], the transcription cofactor PC4 [16] and the oncosuppressor p53 [17]. Rep78 also interacts with and inhibits the catalytic subunit of cyclic AMP-dependent protein kinase A and its homolog PRKX [18]. Other HBV promoters might also be also involved in Rep78-mediated inhibition. Further studies are required to gain a better understanding of the metabolism, toxicology and pharmacokinetic parameters of Rep78.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (30500425), National High Technology Research and Development Program of China (2006AA02A410) and the Major State Basic Research Development Program (2007CB512802).

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