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trans-Complementation of HBV rtM204I mutant replication by HBV wild-type polymerase

Richard A. Heipertz Jr.[a,1], Jason L. Starkey[a,1], Thomas G. Miller[a], Jianming Hu[a], Harriet C. Isom[a,b,*]

[a] Department of Microbiology and Immunology, Milton S. Hershey Medical Center, The Penn State College of Medicine, 500 University Drive, P.O. Box 850, Hershey, PA 17033, USA
[b] Department of Pathology, Milton S. Hershey Medical Center, The Penn State College of Medicine, 500 University Drive, P.O. Box 850, Hershey, PA 17033, USA

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ABSTRACT

The function of the hepatitis B virus (HBV) wild-type (WT) polymerase (pol) expressed alone or in the context of the intact genome when interacting with HBV rtM204I in HepG2 cells was compared. We show that WT pol expression from a packaging-defective RNA can complement defective rtM204I pol activity resulting in increased levels of HBV replicative intermediates (RI). Analysis of the genetically marked genomes showed that this restoration resulted from *trans*-complementation, rather than recombination. In contrast, we demonstrate that enhanced levels of total HBV RI observed when cells were cotransduced with both WT and rtM204I baculoviruses were predominantly WT RI. In this case, WT pol was produced from a full-length pregenomic RNA (pgRNA). We conclude that the WT pol has the capacity to *trans*-complement the replication defect of rtM204I; however, when expressed from an authentic pgRNA, as in a mixed infection, pol may not *trans*-complement efficiently.

Introduction

Hepatitis B virus (HBV) causes acute and chronic infections of the liver and is responsible for 1.2 million deaths annually. Approximately 0.5% of acute infections terminate in fatal, fulminant hepatitis. The WHO currently estimates that 350 million people are chronically infected with HBV.

The approved agents for the treatment of HBV chronically infected individuals include α-interferon and five nucleoside/tide analogues: lamivudine (3TC, Epivir-HBV), adefovir dipivoxil (Hepsera), entecavir (Baraclude), telbivudine (Tyzeka), and tenofovir (Viread). Lamivudine treatment has been very useful in reducing HBV DNA levels in patient sera and shows very limited side effects (Dienstag et al., 1999; Jarvis and Faulds, 1999; Santantonio et al., 2000); however, the emergence of drug-resistant mutants occurs at high frequency, (Allen et al., 1998; Bartholomew et al., 1997; de Man et al., 1999; Honkoop et al., 1997; Liaw et al., 1999; Peters et al., 1999) with the incidence of resistance after 1 year of treatment ranging from 17% (Chang et al., 2004) to 32% (Lai et al., 2003; Mauss and Wedemeyer, 2008). The most common mutations occur within the catalytic YMDD motif of the HBV pol, resulting in the replacement of methionine with either valine or isoleucine at amino acid 204 (Allen et al., 1998; Ono-Nita et al., 1999).

In vitro, the rtM204I lamivudine-resistant mutant replicates to significantly lower levels compared to wild-type (WT) HBV (Fu and Cheng, 1998; Gaillard et al., 2002; Melegari, Scaglioni, and Wands, 1998; Ono-Nita et al., 1999). We recently reported that the rtM204I mutant has a defect in replication, resulting in diminished levels of HBV intracellular replicative intermediates (RI) and extracellular (EC) DNA compared to WT HBV after day 5 post-transduction (p.t.) with HBV recombinant baculovirus (Heipertz et al., 2007). In contrast, *in vivo*, as drug-resistant mutants emerge, there is an increase in viral load and serum alanine aminotransferase levels as well as a deterioration of liver histology. Indeed, lamivudine-resistant mutants have also been associated with death in several case studies (Kagawa et al., 2004; Kim et al., 2001; Suzuki et al., 2007; Wang et al., 2002).

Interestingly, it appears that the rtM204I and rtM204V mutants can preexist in naïve patients (Pallier et al., 2006; Zhang et al., 2003). This is most likely due to the fact that HBV pol lacks proofreading function, leading to a relatively high frequency of mutations/site/year (Girones and Miller, 1989). Therefore, WT and rtM204I HBV viruses can potentially be present in the same hepatocyte, which could lead to an interaction between WT virus and lamivudine-resistant mutants. The interplay of HBV viral components, specifically, the rescue of a pol-deficient mutant virus by the expression of a WT pol *in trans*, has

* Corresponding author. Department of Microbiology and Immunology, Milton S. Hershey Medical Center, The Penn State College of Medicine, 500 University Drive, P.O. Box 850, Hershey, PA 17033, USA. Fax: +1 717 531 5103.
E-mail address: hisom@psu.edu (H.C. Isom).
[1] These authors contributed equally to this work.

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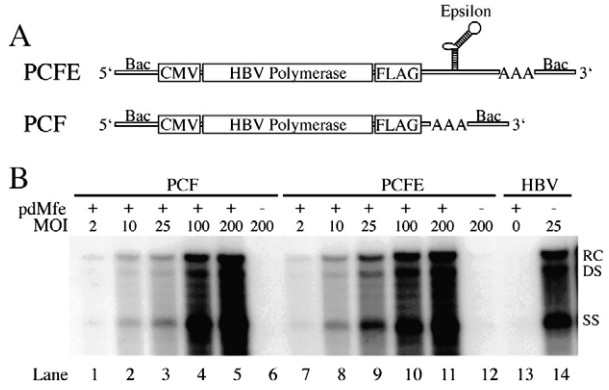

Fig. 1. Generation and screen of HBV pol-expressing recombinant baculoviruses. (A) Schematic representation of CMV promoter-driven, C-terminally FLAG-tagged HBV pol-expressing recombinant baculoviruses PCF and PCFE. PCF and PCFE differ only in the presence of the 3′ epsilon sequence contained within PCFE. (B) Southern blot analysis of HBV RI DNA extracted from HepG2 cells transfected with 3 μg of the replication-deficient HBV plasmid, pdMfe, 4 days p.t. with pol-expressing recombinant baculoviruses PCF or PCFE at various MOIs. HBV RI DNA extracted from HBV recombinant baculovirus-transduced HepG2 cells 4 days p.t. was included as a positive control. MOI, multiplicity of infection (pfu/cell); RC, relaxed-circular; DS, double-stranded linear; SS, single-stranded; +, presence of pdMfe; −, absence of pdMfe.

been demonstrated previously. *trans*-Complementation has been demonstrated when the mutant virus encodes a pol containing a missense codon, rendering the viral pol nonfunctional for RNA encapsidation (Blum et al., 1991), and when the mutant virus encodes a nonsense mutation, preventing the expression of the pol (Bartenschlager, Junker-Niepmann, and Schaller, 1990; Chiang et al., 1990; Junker-Niepmann, Bartenschlager, and Schaller, 1990; Okamoto et al., 1993; Radziwill, Tucker, and Schaller, 1990; Yaginuma et al., 1987). Other mutant viral proteins such as the core protein (HBcAg) and the surface protein (HBsAg) can also be rescued through *trans*-complementation (Chiang et al., 1990; Junker-Niepmann, Bartenschlager, and Schaller, 1990; Okamoto et al., 1993; Radziwill, Tucker, and Schaller, 1990; Yaginuma et al., 1987).

The goal of the current study was to compare the function of the HBV WT pol expressed alone or in the context of the intact HBV genome in HepG2 cells transduced with the lamivudine-resistant rtM204I. To achieve this goal, we took advantage of the recombinant baculovirus-mediated HBV transduction of HepG2 cells, which allows highly efficient transduction of WT and mutant HBV genomes or individual genes and detailed analyses of all post-entry steps of HBV replication.

Results

Generation of recombinant baculovirus that is expressing functional HBV pol

Two WT HBV pol recombinant baculoviruses designated PCF and PCFE were generated (Fig. 1A). PCF and PCFE are C-terminally FLAG-tagged, CMV promoter-driven, HBV pol-expressing recombinant baculoviruses lacking the 5′ epsilon and differing from each other only in the presence of the 3′ epsilon contained within PCFE. The ability of PCF and PCFE to express a functional HBV pol was tested in the context of the replication-deficient HBV expression plasmid, pdMfe, which lacks a functional pol. Twenty-four hours following transfection with 3 μg of pdMfe, HepG2 cells were transduced with pol-expressing baculoviruses, PCF or PCFE, at various MOIs. HBV RI were harvested from infected cells 4 days p.t. and visualized by Southern blot analysis. As shown in Fig. 1B, both PCF and PCFE were able to rescue the replication-deficient pdMfe in a MOI-dependent manner up to a MOI of 100 pfu/cell. The efficacy of PCF and PCFE appear equal, suggesting that the presence or absence of an epsilon sequence does not influence the ability of the pol to rescue the deficient pdMfe. It is important to note that PCF and PCFE (Fig. 1B, lanes 6 and 12, respectively), as well as pdMfe (Fig. 1B, lane 13) are incapable of producing a detectable signal when individually expressed in HepG2 cells, indicating that these constructs are replication-deficient. Due to the unique arrangement of the HBV genome, PCF and PCFE could have the potential to produce HBsAg; however, neither PCFE nor PCF produced detectable levels of HBsAg (data not shown).

pdMfe is a HBV expression plasmid that is replication-deficient due to a frameshift mutation within the pol open reading frame caused by a four-nucleotide insertion, which also results in the loss of the single Mfe I restriction enzyme site present in the HBV genome. To determine whether the rescue of the replication-deficient pdMfe was indeed due to *trans*-complementation by the recombinant baculoviruses PCF and PCFE and not due to recombination of the introduced HBV DNAs, restriction enzyme digestions of the HBV RI were conducted (Fig. 2). Equal concentrations of RI DNA from either WT HBV recombinant baculovirus-transduced HepG2 cells and PCF or PCFE recombinant baculovirus-transduced, pdMfe transfected HepG2 cells were digested with EcoR I and Mfe I or undigested and visualized by Southern blot analysis. As shown in Fig. 2, RI DNA from WT HBV baculovirus-transduced HepG2 cells was digested with both Mfe I and EcoR I, resulting in the conversion of the relaxed-circular (RC) RI species to the double-stranded linear (DS) form and a downward shift in the digested DS form due to the single presence of each restriction enzyme site within the WT HBV genome. However, RI DNA from PCF and PCFE baculovirus-transduced, pdMfe transfected HepG2 cells was only digested by EcoR I, indicating that the single Mfe I site contained within the HBV genome was not restored. Taken together, the data indicate that both PCF and PCFE express a functional HBV pol that is capable of complementing the replication-deficient pdMfe *in trans*. In addition, the epsilon sequence does not need to be present in the construct to produce functional HBV pol. The remaining experiments were carried out with only the 3′ epsilon-containing construct, PCFE.

Effect of the WT pol construct PCFE on the replication of the lamivudine-resistant mutant rtM204I

The goal of these experiments was to examine the effect of the WT pol construct PCFE on the replication of the lamivudine-resistant mutant rtM204I. Examining the effect of the WT pol construct PCFE on the replication of the WT HBV was included as a control. HepG2 cells were seeded in 60-mm dishes, grown for 16 to 24 h, and then transduced with 100 pfu/cell of WT or rtM204I HBV recombinant baculovirus. Cells were fed every-other day and maintained until day

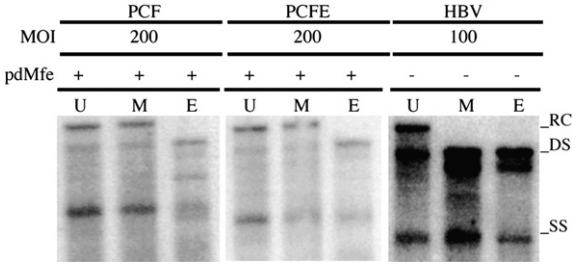

Fig. 2. *trans*-Complementation of a replication-deficient HBV expression plasmid by HBV pol-expressing recombinant baculoviruses. Southern blot analysis of restriction enzyme digested HBV RI DNA. HepG2 cells were transduced with HBV pol-expressing recombinant baculoviruses, PCF or PCFE, at a MOI of 200 pfu/cell 24 h post-transfection with 3 μg of the replication-deficient HBV expression plasmid, pdMfe. HepG2 cells were transduced with WT HBV recombinant baculovirus at a MOI of 100 pfu/cell as a positive control. Four days p.t., HBV RI DNA was extracted and equal concentrations were left undigested or digested with EcoR I or Mfe I at 37 °C for 1 h and visualized by Southern blot analysis. U, Undigested; E, EcoR I digested; M, Mfe I digested, MOI, multiplicity of infection (pfu/cell); RC, relaxed-circular; DS, double-stranded linear; SS, single-stranded; +, presence of pdMfe; −, absence of pdMfe.