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ScienceDirect
Virology 353 (2006) 443–450
VIROLOGY
www.elsevier.com/locate/yviro

The insertion domain of the duck hepatitis B virus core protein plays a role in nucleocapsid assembly

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Received 12 April 2006; returned to author for revision 11 May 2006; accepted 6 June 2006
Available online 11 July 2006

Abstract

Synthesis of hepadnaviral DNA is dependent upon both the viral DNA polymerase and the viral core protein, the subunit of the nucleocapsids in which viral DNA synthesis takes place. In a study of natural isolates of duck hepatitis B virus (DHBV), we cloned full-length viral genomes from a puna teal. One of the clones failed to direct viral DNA replication in transfected cells, apparently as a result of a 3 nt inframe deletion of histidine 107 in the core protein. Histidine 107 is located in the center of a predicted helical region of the "insertion domain", a stretch of 45 amino acids which appears to be at the tip of a spike on the surface of the nucleocapsid. The mutation was introduced into a well-characterized strain of DHBV for further analysis. Core protein accumulated in cells transfected with the mutant DHBV but was partially degraded, suggesting that it was unstable. Assembled nucleocapsids were not detected by capsid gel electrophoresis. Interestingly, the mutant protein appeared to form chimeric nucleocapsids with wild-type core protein. The chimeric nucleocapsids supported viral DNA replication. These results suggest that the insertion domain of the spike may play a role either in assembly of stable nucleocapsids, possibly in formation of the dimer subunits, or in triggering nucleocapsid disintegration, required during initiation of new rounds of infection.

Keywords: Duck hepatitis B virus; Nucleocapsid assembly; Insertion domain

Introduction

Hepadnaviruses are small enveloped DNA viruses that replicate via reverse transcription (Seeger and Mason, 2000). The family is divided into two genera, *Orthohepadnavirus* and *Avihepadnavirus*, with host specificity for mammals and birds, respectively. Reverse transcription takes place following packaging of viral pregenomic RNA, polymerase and host-encoded chaperones (Hu and Seeger, 1996; Hu et al., 1997) into icosahedral viral nucleocapsids, assembled from dimers of the viral core protein (Zhou and Standring, 1992). The core protein of avian hepadnaviruses is longer than that of HBV (262 aa versus 183) due to an extra 45 contiguous amino acids (aa 86 to aa 130) (Bringas, 1997), defined as an insertion domain, as well as additional amino acids in the arginine-rich C-terminal, nucleic acid binding domain (Beames and Lanford, 1993; Hatton et al., 1992; Kock et al., 1998; Nassal, 1992; Yu and Summers, 1991).

At the 24 amino acid carboxy-terminus of the DHBV core protein, there are four sites that may be phosphorylated (T239, S245, S257 and S259) (Yu and Summers, 1994b). Dephosphorylation at these sites takes place as the DHBV nucleocapsids accumulate double-stranded DNA and is essentially complete in mature virions. This observation implies that dephosphorylation is associated with nucleocapsid maturation. A maturation event is implied by the fact that virus is preferentially assembled from nucleocapsids containing partially double-stranded DNA rather than single-stranded DNA or pregenomic RNA (Perlman et al., 2005; Pugh et al., 1989; Rabe et al., 2003). The DHBV core protein is also phosphorylated at other amino acids. It has been suggested that phosphorylation at threonine 174 triggers disintegration of the viral nucleocapsid and delivery of viral DNA into the nucleus (Barrasa et al., 2001).

In contrast to the arginine-rich carboxy-terminus, the amino-terminus is involved in assembly of the nucleocapsid

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0042-6822/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.06.004

shell. When the arginine-rich region is deleted, nucleocapsids are still assembled, but packaging of viral RNA does not take place (Kock et al., 1998; Yang et al., 1994). The structure of the HBV nucleocapsid has been derived by cryoEM and, at higher resolution, by X-ray crystallography (Bottcher et al., 1997; Conway et al., 1997; Wynne et al., 1999). The building block is a core protein homodimer (Zhou and Standring, 1992). The regions from amino acids 50–73 and 79–110 of the monomer fold into α helices that fold back on each other to form a hairpin with a short loop between amino acid 73 and 79. Interactions between the first helix of each monomer stabilize the homodimer. The resulting four helix structure forms a spike projecting from the nucleocapsid shell, with the loop regions at the tip of the spike. Large insertions near the tip of the spike of HBV, at amino acid 80, typically do not block nucleocapsid assembly, while mutations in the alpha helices forming the sides of the spike can block assembly (Beames and Lanford, 1995; Brown et al., 1991; Newman et al., 2003). Early studies suggested that the tip of the spike interacted with viral envelope proteins during virus formation because a peptide that bound to the tip blocked virion formation (Bottcher et al., 1998). However, a later genetic analysis suggested that amino acids involved in interactions between the nucleocapsid and envelope proteins and critical for virion formation mapped to the base of the spike (Ponsel and Bruss, 2003). Thus, the function of the distal portion of the spikes remains unclear.

Relative to HBV, DHBV contains a 45 amino acid insertion which, based on amino acid alignments (Bringas, 1997), would be located at a position homologous to the tip of the spike of the HBV nucleocapsid. Low resolution cryoEM of DHBV nucleocapsids is consistent with the placement of the insertion domain in the spikes (Kenney et al., 1995). Model building using the crystal structure of the HBV nucleocapsid as a template suggests a location at the tip of the spike, forming the loop of a stem loop structure with α helices as the stem (Thermet et al., 2004). In agreement with the idea that the insertion domain is exposed at the tip of the spike, amino acids 99–112 in the insertion domain, with a predicted α helical structure (Bringas, 1997; Thermet et al., 2004), are recognized by antisera from chronically infected ducks (Thermet et al., 2004). The function of the DHBV insertion domain is unknown.

In a study of natural isolates of avian hepadnaviruses, we cloned from the puna teal a virus mutant that was defective in viral DNA synthesis as the result of a histidine deletion (aa 107) in the insertion domain of the core protein (Guo et al., 2005). In order to characterize this mutation further, we introduced it into the genome of a laboratory strain of DHBV, DHBV-16 (Mandart et al., 1984), and demonstrated that the defect in DNA synthesis was due to a failure to form nucleocapsids. Substitutions of H107 that conserved the ability to form an α helix had no effect on nucleocapsid formation, while a proline substitution, which would disrupt the α helix, blocked nucleocapsid assembly. Thus, the insertion domain of the DHBV spike either provides information essential for formation of nucleocapsids or encodes a signal, activated by the H107 deletion, for disassembly of nucleocapsids (e.g., when wt virus enters the nuclear basket; Rabe et al., 2003). Interestingly, core protein with the H107 deletion appeared to form viable, chimeric nucleocapsids when co-expressed with wtCore protein.

Results

HisDHBV is replication defective in LMH cells

DHBV DNA replication intermediates were not detected in LMH cells transfected with DHBV DNA containing the histidine 107 deletion mutation in the core protein (HisDHBV). Replication was rescued by trans-complementation with wild-type DHBV core protein (wtCore), indicating that the mutation was not trans-dominant (Figs. 1, 2A). It has been reported that viral DNA synthesis may destabilize nucleocapsids assembled from mutated core proteins (Kock et al., 1998). We therefore investigated the possibility that the DNA within nucleocapsids formed by mutant core protein was destroyed by the DNaseI digestion used to eliminate plasmid DNA prior to extraction of viral DNA from nucleocapsids. Extractions were performed without DNaseI treatment, and samples were incubated with *Dpn*I to digest the input plasmid DNA, which is methylated. The results were the same as with DNaseI treatment (Fig. 2A).

To determine if the defect in DNA synthesis might be due to a failure to package pregenomic RNA into nucleocapsids, northern blot analysis was carried out on total cellular RNA and on RNA isolated from viral nucleocapsids present in transfected cells. As shown in Fig. 2B, packaging of pregenomic RNA was not detected in cells transfected with HisDHBV. This defect could be rescued by co-transfection with a plasmid expressing the wtDHBV core protein. The slightly smaller size of the pgRNA within nucleocapsids than the core/pol mRNAs has been previously reported and attributed to digestion of the 3′ end by ribonucleases during nucleocapsid isolation (Ostrow and Loeb, 2004). The smear of signal below the pregenome presumably reflects partial degradation of pgRNA by the viral RNaseH during elongation of minus strand DNA by reverse transcription.

The accumulation of mutant core protein in transfected cells was assayed by immunoblotting. Partial degradation was evident in the HisCore transfected cells (Fig. 3), suggesting that the mutant protein may be less stable than the wt, perhaps due to a failure to assemble into dimers or nucleocapsids. The presence of multiple species of mutant protein that, like the wild-type core protein, migrates slower than the major band suggested that the mutant core protein is phosphorylated (Pugh et al., 1989).

The mutant core protein forms functional chimeric nucleocapsids when co-expressed with wtCore protein

To see if the mutant core protein could assemble into nucleocapsids, lysates of the transfected cells were subjected to capsid gel electrophoresis in agarose. The nucleocapsids