Rapid Communication

The hepatitis *delta* virus RNA genome interacts with the human RNA polymerases I and III

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ABSTRACT

The hepatitis *delta* virus (HDV) relies on human transcriptional machinery for its replication and transcription. Although the involvement of RNA polymerase II in HDV RNA biosynthesis is established, the contribution of additional polymerases remains uncertain. Here, we demonstrate the interaction of both RNA polymerase I and III with HDV RNA, both *in vitro* and in human cells. Binding of these polymerases occurs near the terminal stem-loop domains of both polarities of the HDV RNA genome. Based on interactions of HDV RNA with numerous host polymerases, our results suggest a higher level of complexity of HDV biology than previously envisioned.

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Introduction

The hepatitis *delta* virus (HDV) is composed of a single-stranded circular RNA molecule of approximately 1700 nucleotides that folds into a rod-like structure and contains a single open reading frame (Taylor, 2006). Because HDV does not encode an RNA-dependent RNA polymerase, it relies on host polymerase(s) for its replication and transcription. It is acknowledged that RNA polymerase II (RNAP II) is involved in HDV replication based on the sensitivity of the process to α-amanitin, and on the post-transcriptional processing of the HDV mRNA (i.e. HDAg mRNA) with a 5′ methylguanine cap and a poly(A) tail (MacNaughton et al., 1991; Fu and Taylor, 1993; Gudima et al., 1999; Lai, 2005; Taylor, 2006). Recently, we confirmed the interaction of RNAP II with HDV RNA both *in vitro* and *in vivo* and demonstrated the binding of this polymerase to the terminal stem-loop domains of both polarities of the HDV genome (Greco-Stewart et al., 2007). The involvement of RNAP I in HDV biology has been suggested based on the resistance of antigenomic RNA production to higher doses of α-amanitin, on the co-localization of RNAP I with antigenomic HDV RNA, on the interaction of HDAg-S with components of the RNAP I transcription factor SL1, and on the inhibition of antigenomic RNA synthesis *in vitro* by α-SL1 antibody (Modahl et al., 2000; Macnaughton et al., 2002; Li et al., 2006). However, no direct interaction between RNAP I and HDV RNA has been reported, and a role for RNAP III in HDV replication has never been investigated. In this study, we

report the association of both RNAP I and RNAP III components with HDV RNA, both *in vitro* and *in vivo*. Our results indicate the recruitment of the two RNAPs by both polarities of HDV RNA and the possible involvement of the TATA-binding protein (TBP) in these processes. Our data suggest a mechanism of HDV replication involving all three human RNAPs.

Results

HDV RNA interacts with components of RNAP I and RNAP III in cells replicating HDV

Because RNAPs must bind their templates to transcribe, we investigated whether components of RNAP I and/or RNAP III associate with the HDV RNA genome in cells replicating HDV RNA. To establish these interactions, we took advantage of a ribonucleoprotein immunoprecipitation assay (RIPA), which has been successfully used to indicate HDAg, PSF, and RNAP II interaction with HDV RNA (Niranjanakumari et al., 2002; Greco-Stewart et al., 2006, 2007). HeLa cells were co-transfected with a dimeric HDV RNA genome and a plasmid expressing HDAg-S. Four days post-transfection, the ribonucleoproteins were cross-linked with formaldehyde and immunoprecipitated using antibodies against either TAF I p95/110 (TAF1) or RNAP III polypeptide K (POLR3K). TAF1 is a component of the transcription factor SL1 which directs the assembly of initiation complexes at RNAP I promoters. POLR3K is one of the core subunits of RNAP III. Following immunoprecipitation, the cross-links were reversed by heating the samples and the recovered nucleic acids were subjected to RT-PCR

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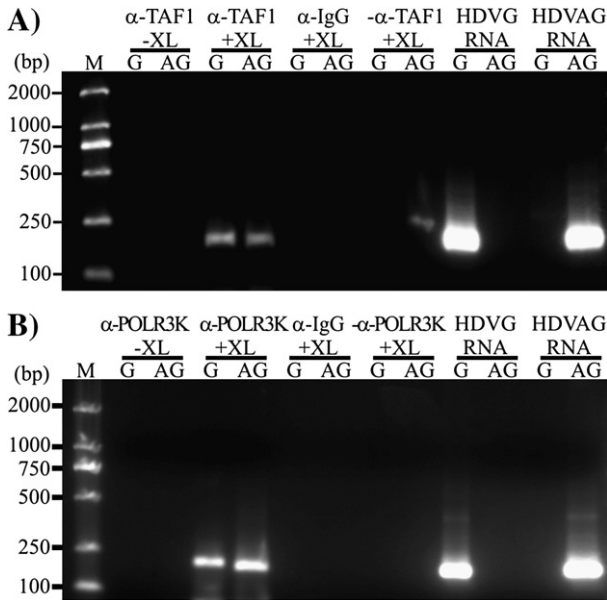
Fig. 1. Interaction of RNAP I and RNAP III components with HDV RNA in HeLa cells. HeLa cells replicating HDV RNA were used for the preparation of cross-linked lysates for co-immunoprecipitation using either α-TAF1 (A), α-POLR3K (B), α-IgG, or no antibody. Experiments using non-cross-linked lysates are also presented. G and AG lanes contain RT reactions performed using primers complementary to either genomic or anti-genomic HDV RNA corresponding to the R199 region. HDVG and HDVAG RNA lanes are controls using *in vitro* transcribed HDV RNA of genomic and antigenomic polarity, respectively. Lanes M contain GelPilot Mid Range DNA ladder (Qiagen).

using primers to generate a 232 bp cDNA fragment corresponding to the R199 region (see Fig. 2B) (Greco-Stewart et al., 2007).

HDV RNA associated with both TAF1 and POLR3K in cross-linked HeLa cells. The interactions were specific since HDV RNA failed to co-immunoprecipitate in the absence of formaldehyde treatment, without antibody, or in the presence of a non-related antibody (i.e. α-IgG; Fig. 1). In addition, the primers used in the RT reactions allowed differentiation between the polarities of HDV RNA, as demonstrated when *in vitro* transcribed HDV RNAs were subjected to RT-PCR (Fig. 1, HDVG RNA and HDVAG RNA lanes) (Greco-Stewart et al., 2007). Since both polarities of HDV RNA were recovered when the cross-linked samples were immunoprecipitated with these antibodies, we conclude that both TAF1 and POLR3K associate with both polarities of HDV RNA within HeLa cells.

RNAP I and RNAP III components bind close to the terminal stem-loop domains of HDV

To determine the regions of the HDV genome to which subunits of RNAP I and RNAP III bind, various portions of the HDV genome were transcribed *in vitro*, radiolabelled, and subjected to co-immunopre-cipitation using α-TAF1 and α-POLR3K antibodies (Fig. 2). Fig. 2A shows a representative experiment using a segment of the right terminal stem-loop domain of genomic polarity (i.e. R199G). R199G associated with TAF1 in the presence of HeLa NE proteins. In the absence of antibody or in the presence of a non-related antibody (i.e. α-IgG), R199G failed to associate with TAF1. TAF1 binding was not hindered by the addition of a 50-fold excess of either P11.60 or poly(A) RNA, although a 50-fold excess of unlabelled homologous competitor prevented association. P11.60 is a small RNA species that folds into a hairpin (Pelchat and Perreault, 2004), thus providing an excellent competitive RNA to test for non-specific affinity of the proteins to double-stranded RNA or stem-loop secondary structures.

Co-immunoprecipitations of the terminal stem-loop domains (i.e. R199G, R199AG, L213G, and L213AG) and the central regions (i.e. CENTRG and CENTRAG) of both polarities of HDV RNA were performed

using both antibodies. We observed interaction of TAF1 and POLR3K to fragments containing the terminal stem-loop domains of the HDV genome of both polarities (Fig. 2B) in a manner similar to what was observed for RNAP II interaction (Greco-Stewart et al., 2007). These data suggest that not only RNAP II, but also RNAP I and RNAP III, might be involved in HDV biology through specific association with the extremities of both polarities of the HDV RNA genome.

To further refine the regions interacting with both RNAP I and RNAP III and to determine if the RNA features bound by those RNAPs are identical to those used by RNAP II, two small HDV-derived transcripts reported to be able to associate with RNAP II were synthesized and were used in the co-immunoprecipitation reactions (Greco-Stewart et al., 2007). Specifically, R38G and L48AG, which correspond to 38 and 48 nt RNAs located at the tip of the right genomic and the left antigenomic hairpin domains, respectively, were tested. These two small RNA molecules were not able to co-immunoprecipitate using either α-TAF1 or α-POLR3K antibodies (Fig. 2B), suggesting either that those proteins do not bind to the tip of the rod-like structure of HDV RNA or that additional features present on the larger RNA species are required.

RNAP I and RNAP III binding to HDV RNA is independent and involves the TATA-binding protein

The binding of the three RNAPs to the same regions of HDV RNA suggests a common mechanism of RNA recognition, which might involve a shared subunit. Since TBP is common to three RNAPs during DNA promoter recognition (Goodrich and Tjian, 1994) and this protein was suggested to be involved in HDV RNA promoter recognition by RNAP II (Abrahem and Pelchat, 2008), we speculated that TBP might
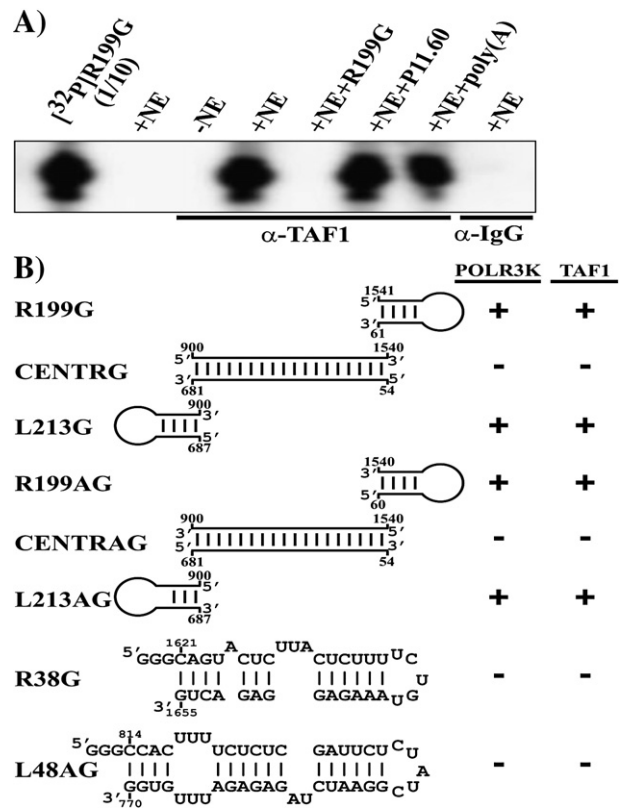
Fig. 2. Association of TAF1 and POLR3K with HDV-derived RNAs. (A) Co-immunopre-cipitations of radiolabelled R199G with TAF1 in HeLa NE. 50-fold excess non-radioactive R199G, poly(A) RNA, or P11.60 (Pelchat and Perreault, 2004) were used as competitors. (B) Compilation of the interaction of various HDV-derived RNAs with both TAF1 and POLR3K.

be present during the binding of HDV RNA to both RNAP I and RNAP III. RIPA was initially performed to determine the association of TBP with HDV RNA in HeLa cells. As shown in Fig. 3A, TBP associated with both polarities of HDV RNA *in vivo*. HDV RNA binding to TBP was specific since the RIPA was only successful in cross-linked HeLa cells and failed in the absence of α-TBP antibody or in the presence of a non-related antibody. However, this assay did not discriminate the polymerase involved.

To verify the presence of TBP in both RNAP I and RNAP III complexes independently of one another and of RNAP II, we performed RNA affinity chromatography followed by immunoprecipitation and Western analysis. For this purpose, we selected R199G, which binds to all three RNAPs (Fig. 2B and Greco-Stewart et al., 2007), and added an RNA tag with an affinity for streptavidin (Srisawat and Engelke, 2002). We used a tagged P11.60 as a negative control since P11.60 does not bind human RNAPs (this work and Greco-Stewart et al., 2007). The tagged RNAs were immobilized on streptavidin-agarose beads and incubated with HeLa NE. Following washes, the RNP complexes were eluted by competition with molecular excess of *d*-biotin, immunoprecipitated with either α-TAF1 or α-POLR3K antibody, and the presence of the tagged RNAs was confirmed by PAGE (data not shown). The samples were then subjected to SDS-PAGE, and Western blotting was performed to determine the composition of these complexes using specific antibodies.

TAF1 but not POLR3K was detected in complexes immunoprecipitated using the α-TAF1 antibody (Fig. 3B). Conversely, complexes immunoprecipitated using the α-POLR3K antibody contained POLR3K but not TAF1. However, TBP was common to both complexes. Neither protein was detected when S1-R199G was substituted with S1-P11.60, indicating the specificity of these protein–RNA interactions (Fig. 3B). To further demonstrate that RNAP II was not involved in the formation of these complexes, the TAF1 and POLR3K complexes were each probed with an antibody against RNAP II carboxyl-terminal domain (Fig. 3B). RNAP II was not present in either of these complexes, indicating that HDV RNA binding to both TAF1 and POLR3K is independent of RNAP II binding. These results demonstrate the binding of TAF1 and POLR3K complexes to HDV RNA, either independently or through a common subunit, and suggest the involvement of TBP in both RNAP I and RNAP III binding.

Discussion

Using antibodies directed against components of both RNAP I and RNAP III, we have further implicated RNAP I in HDV replication and strongly suggested a role for RNAP III in this process. Combined with the established role of RNAP II, the association of both RNAP I and RNAP III with HDV RNA suggests the involvement of the three human RNAPs in HDV biology. Interestingly, the three RNAPs bind near or at the terminal stem-loop domains of HDV (this work and Greco-Stewart et al., 2007). TBP is common to the three RNAPs: it is found in the SL1 complex of RNAP I, the TFIID complex of RNAP II, and the TFIIIB complex of RNAP III, as well as the SNAP$_c$ complex used in transcription of snRNAs by both RNAPs II and III (Goodrich and Tjian, 1994). TBP can recognize both TATA and TATA-less promoters dependent on accessory transcription factors, and its affinity for DNA templates is mediated by electrostatic interactions with the phosphate backbone (Bewley et al., 1998), which could account for its ability to bind HDV RNA despite the absence of known DNA promoter features. Because TBP was found in the RNAP complexes, it is tempting to suggest that this shared polymerase subunit might mediate binding of all three RNAPs to an HDV template. In addition, we recently reported that TBP binding occurs at the tip of the rod-like structure of HDV (i.e. binding sites are within R38G and L48AG; Abrahem and Pelchat, 2008). Because additional features present on the larger RNA species might be required for both RNAP I and RNAP III association, it is likely that accessory host factors might bind to these features and provide a link between the RNAPs and the HDV-derived RNA promoters at the initiation phase of transcription.

RNAP I was reported to be implicated in the synthesis of antigenomic, but not genomic HDV RNA (Modahl et al., 2000; Macnaughton et al., 2002; Li et al., 2006). In addition, a role for RNAP III in HDV replication has never been reported. It is therefore possible that some of the associations observed in this study might not lead to transcription initiation. Because there is currently no suitable assay to directly study the transcription of RNAPs on HDV RNA templates, the roles of these RNAPs in the HDV life cycle will be difficult to address. The development of a sensitive and reproducible transcription system using HDV RNAs will be crucial to allow a straightforward testing of the relevance of our findings. Nevertheless, the three human RNAPs are capable of independent binding to the terminal stem-loop domains of HDV and each RNAP complex contains TBP. Taken together, this study provides the foundation to further investigate HDV biology by suggesting a role for the three human RNAPs, where each polymerase complex might have a specific function during HDV replication and transcription.

Materials and methods

RNA synthesis

HDV-derived RNAs and P11.60 were synthesized by run-off transcription using T7 RNA polymerase, as previously described (Pelchat and Perreault, 2004; Greco-Stewart et al., 2007). When needed, S1 aptamer was encoded in one of the primers to generate a streptavidin tag at the 3′ end of the RNAs (Srisawat and Engelke, 2002).

Ribonucleoprotein immunoprecipitation assay (RIPA)

HeLa cells were transfected with 6 μg of pCDNA3-AgS and 10 μg of dimeric HDV genomic RNA (Greco-Stewart et al., 2007). ~10^6 cells
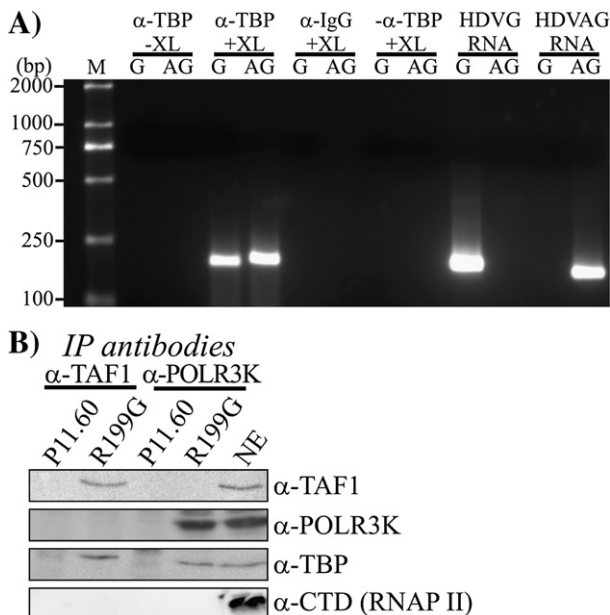
Fig. 3. Presence of TBP in both RNAP I and RNAP III complexes on HDV RNA. (A) Interaction of TBP with HDV RNA within HeLa cells, as described in Fig. 1 using α-TBP. (B) Mutual exclusivity of RNAP complexes and the involvement of TBP. S1-R199G and S1-P11.60 RNA were immobilized on streptavidin-agarose beads and incubated with HeLa NE. Following washes, the RNP complexes were eluted by competition with a molecular excess of *d*-biotin, and immunoprecipitated with either α-TAF1 or α-POLR3K antibodies. The proteins were detected using specific antibodies.

were treated with 1% formaldehyde, disrupted by sonication, and co-immunoprecipitated with the Protein-G Immunoprecipitation Kit (Sigma-Aldrich) using α-TAF1 (Santa Cruz), α-POLR3K (U.S. Biologicals), α-TBP (Upstate), or mouse α-IgG (Sigma-Aldrich) antibodies in RIPA buffer (50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl), as previously described (Niranjanakumari et al., 2002; Greco-Stewart et al., 2007). Samples were then eluted, treated with DNAse I, phenol/chloroform extracted, ethanol precipitated, and reverse transcribed using strand-specific primers (Greco-Stewart et al., 2007). Samples were amplified by 35 cycles of PCR using the same primers. Products were resolved on 2% agarose gels and visualized by SYBR green staining. These assays were performed at least twice.

Co-immunoprecipitation analysis

The protein-G Immunoprecipitation Kit was used with ∼50 μg of HeLa Nuclear Extract (NE; Promega), 5 μg of antibody, and ∼1 pmol of 5′-end radiolabelled RNA, as previously described (Greco-Stewart et al., 2007). Competitors included 50-fold molar excess of poly(A) RNA, P11.60, or unlabelled homologous RNA. Samples were incubated for 16 h at 4 °C with agitation, washed five times with RIPA buffer, and eluted with acrylamide loading dye (Sambrook et al., 1989). RNAs were separated on denaturing polyacrylamide gels (PAGE) and results visualized by phosphor-imager scanning (Molecular Dynamics). These assays were performed at least twice.

Affinity purification of ribonucleoprotein complexes

30 μl of pre-washed streptavidin agarose beads (Invitrogen) was incubated with ∼200 μg of HeLa NE and 1 nmol of RNA (either S1-R199G or S1-P11.60) in 50 mM HEPES (pH 7.4), 10 mM MgCl$_2$, and 100 mM NaCl for 1 h at 4 °C, followed by a 3 hour wash in the same buffer. Samples were eluted by the addition of 5 mM *d*-biotin (Sigma-Aldrich) and then subjected to co-immunoprecipitation, as above. SDS loading dye was added to elute the samples, which were subjected to SDS-PAGE and Western blotting using the One Hour Complete Western Kit (Genscript) using the antibodies described above, or one directed against RNAP II carboxyl-terminal domain (Upstate).

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