Multiple genomic sequences of hepatitis delta virus are associated with cDNA promoter activity and RNA double rolling-circle replication

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To understand how DNA-dependent RNA polymerase II (pol II) recognizes hepatitis delta virus (HDV) RNA as a template, it is first necessary to identify the HDV sequence that acts as a promoter of pol II-initiated RNA synthesis. Therefore, we isolated the pol II-response element from HDV cDNA and examined the regulation by hepatitis delta antigens (HDAs). Two HDV cDNA fragments containing bidirectional promoter activity were identified. One was located at nt 1582–1683 (transcription-promoter region 1, TR-P1) and the other at nt 1223–1363 (transcription-internal region 5, TR-I5). The promoter activities of these two regions were enhanced by HDAs to differing degrees. Next, the role of these sequences in an HDV cDNA-free RNA replication system was characterized by site-directed mutagenesis. Our data showed that: (i) the AUG codon at the HDAg ORF of HDV RNA (nt 1599–1601) that mutates to UAG (amber stop codon) results in loss of dimeric but not monomeric HDV RNA synthesis. (ii) A 5 nt mutation of TR-P1 (P1-m5, nt 1670–1674) abolishes RNA replication completely. Two-nucleotide-mutated RNA (P1-m2, nt 1662–1663) is able to synthesize short RNAs but not monomeric HDV RNA. (iii) A mutation in 5 nt at the TR-I5 region (I5-m5, nt 1351–1355) also abolishes HDV replication. Mutants with 2 nt mutations (I5-m2, nt 1351–1352) or 3 nt mutations (I5-m3, nt 1353–1355) inhibit HDV dimeric but not monomeric RNA synthesis. Furthermore, large HDAg is expressed in cells transfected with I5-m3 and I5-m2 RNAs and that demonstrate the RNA-editing event in the monomeric HDV RNA. These results provide further understanding of the double rolling-circle mechanism in HDV RNA replication.

INTRODUCTION

Hepatitis delta virus (HDV) has a negative ssRNA genome with a circular conformation. During HDV replication, three different RNA species accumulate: the 1.7 kb antigenomic monomer, the 1.7 kb genomic monomer and the 0.8 kb antigenomic-sense RNA. The first two RNA species are circular RNA and represent the replication products of the HDV RNA genome. The 0.8 kb RNA is polyadenylated in vivo and resembles cellular DNA-dependent RNA polymerase II (pol II) transcripts. It is used for translation of hepatitis delta antigen (HDAG). A rolling-circle mechanism in the mediation of HDV RNA replication has been demonstrated by labelling and kinetic-tracing studies of HDV RNA intermediates (Macnaughton et al., 2002). The dimeric and higher-oligomeric HDV RNA species are true intermediates in HDV replication and serve as the precursors to the monomers. Syntheses of these three unit-length HDV RNAs are carried out under distinct mechanisms of regulation (Hughes et al., 2011; Lai, 2005; Pascarella & Negro, 2011; Taylor, 2006).

HDV contains ribozyme domains for self-cleavage (Ferré-D’Amaré et al., 1998; Sharmeen et al., 1988) and encodes an ORF to produce two different forms of HDAGs: a small form (S-HDAG, 195 aa; 24 kDa) and a large form (L-HDAG, 214 aa; 27 kDa). L-HDAG is synthesized after S-HDAG has been expressed in the viral life cycle. The ORF of L-HDAG is produced by a cellular dsRNA-adenosine deaminase (ADAR1) (Jayan & Casey, 2002; Wong & Lazinski, 2002), as a result of an RNA-editing event (Polson et al., 1996). The activity of S-HDAG is necessary for initiation of HDV RNA replication (Kuo et al., 1989),
whereas L-HDAg acts as a dominant-negative inhibitor (Chao et al., 1990; Lee et al., 1995; Modahl & Lai, 2000) and is essential for virion assembly (Chang et al., 1991; Chen et al., 1992; Sato et al., 2004). Both S-HDAg and L-HDAg have been shown to affect cellular pol II-mediated transcription either positively or negatively using pol II-response elements (Lo et al., 1998; Wei & Ganem, 1998). Apparently, HDAgs are closely associated with the cellular transcription machinery.

Replication of HDV RNA is independent of its helper virus hepatitis B virus and does not involve any DNA intermediates (Chen et al., 1986). In the HDV life cycle, the first RNA molecule that is synthesized is probably the 0.8 kb mRNA species, necessary for encoding S-HDAg. The 5' end of this mRNA starts at nt 1631 (Gudima et al., 2000; Modahl & Lai, 1998). Interestingly, the initiation site of the 0.8 kb antigenomic RNA synthesis is near one end of the rod structure of HDV RNA, where the putative promoter for HDV RNA synthesis is located (Beard et al., 1996; MacNaughton et al., 1993). It has been reported that human RNA polymerases II (Greco-Stewart et al., 2007), I and III (Greco-Stewart et al., 2009) interact with the terminal stem–loop region. RNA pol II forms a pre-initiation complex on this region (Abrahem & Pelchat, 2008). However, whether the initiation site of 1.7 kb antigenomic or genomic HDV RNA synthesis is identical to that of 0.8 kb mRNA transcription is not known. Identification of the authentic RNA promoter locations for all three HDV RNA species is a very important issue and requires further investigation.

The important cis-elements in HDV RNA have been defined by a linker-scanning mutagenesis strategy and the data have suggested that the terminal stem–loop region and multiple regions are required for HDV replication (Wang et al., 1997). Therefore, we sought to investigate the cryptic endogenous promoter sequences in HDV and determine the functions of these sequences in the HDV RNA-replication machinery.

RESULTS

Transcription-promoter region 1 (TR-P1) at the 5' region of the HDAg ORF contains maximal promoter activity

We used the 1.9 kb/pKS plasmid as a cDNA template to screen various fragments of the putative HDV promoter in Huh7 cells using a luciferase reporter gene assay (data not shown). Two fragments containing higher bidirectional promoter activity were observed. One was in the 5'-immediate region of HDAg ORF and was denoted TR-P1, while the other was within HDAg ORF and was denoted transcription-internal region 1 (TR-II) (Fig. 1).

To demonstrate quantitatively that the TR-P1 sequence contains essential promoter activity, a luciferase reporter gene assay was performed. We constructed deletion fragments TR-P2, TR-P3 and TR-P4. In addition, two mutated constructs generated by site-directed mutagenesis of the TR-P1 fragment were denoted P1-m2 (GG1662/1663AA mutation) and P1-m5 (CGCCC to ATATA mutation in nt 1670–1674), respectively (Fig. 1). These two mutated constructs were previously designed by Beard et al. (1996) and reported as M5 (P1-m2) and M1 (P1-m5) constructs with defective HDV replication.

After deletion of the sequence between nt 1654 and 1683 to generate TR-P2 (genomic, G) from TR-P1 (G), the promoter activity was reduced by nearly 50% (from 3.9-fold to 2.0-fold). When this same deletion was carried out in the antigenomic-sense construct to generate TR-P2 antigenomic, AG), the transcriptional activity level was reduced by nearly 60% (from 3.4-fold to 1.4-fold) (Fig. 2a). Mutations of TR-P1 to P1-m2 and P1-m5 also reduced

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**Fig. 1.** Schematic diagram of the locations of HDV cDNA elements. The sequences of TR-P1 and TR-II were two putative HDV cDNA promoters previously examined by the authors. Deletions of the TR-P1 element to form P2, P3 and P4 and the TR-II element to form I2, I3, I4, I5 and I6 were performed consecutively. Mutations of TR-P1 and TR-I5 were generated as P1-m2 (GG1662/1663AA mutation), P1-m5 (CGCCC to ATATA mutation in nt 1670–1674) and I5-m2 (CC1351/1352AA mutation) by site-directed mutagenesis. The numbering system for the HDV sequence was obtained from GenBank accession no. M28267.
the promoter activity significantly compared with TR-P1 (Student’s t-test, P<0.01) (Fig. 2a). Apparently, nt 1654–1683 are necessary for maximal promoter activity of TR-P1. The sequence between nt 1582 and 1591 was deleted sequentially by 10 bp to generate TR-P3 constructs. The first 20 nt from the ORF (nt 1582–1601) were removed completely for generation of the TR-P4 constructs. The TR-P3 constructs exhibited a reduction in transcriptional activity and the promoter activity of the TR-P4 genomic construct was reduced by approximately 50 % when compared with TR-P1. The antigenomic TR-P4 construct exhibited complete loss of promoter activity (Fig. 2a).

Our data revealed that the first 20 nt of the ORF are important for maximal TR-P1 bidirectional promoter activity. Defects in this region have a greater impact on antigenomic promoter activity than on genomic-sense orientation.

To determine whether the promoter of TR-I5 can be further deleted without loss of activity, we generated TR-I6- and TR-I5-mutated constructs (I5-m2, 2 nt mutation, nt 1314–1363) (Fig. 2b). Deletion of nt 1314–1363 and mutation of I5-m2 markedly reduced promoter activity when compared with TR-I5 promoter activity (Student’s t-test, P<0.01) (Fig. 2b). These results showed that the TR-I5 sequence is capable of acting from both orientations for luciferase expression and is a novel sequence that is essential for cDNA promoter activity when compared with the TR-P1 sequence.

Activities of HDV cDNA promoters are positively regulated by S- and L-HDAG

It has been well documented that S-HDAG is required to initiate replication (Kuo et al., 1989) and accumulation of L-HDAG is required to regulate replication (Modahl & Lai, 2000; Moraleda et al., 2000; Sheu & Lai, 2000). How HDAGs regulate pol II activity is still unclear, especially in the interaction between HDAGs and pol II using the HDV sequence as a template. Furthermore, the sequences of TR-P1 and TR-I5 are not the known homologous sequences for interaction with pol II. Therefore, it is possible that regulation of HDAGs with TR-P1 and TR-I5 promoters provides an association with the specific recognition mechanism by pol II. Our results showed that S-HDAG enhances TR-P1 and TR-I5 activities in both the G (Fig. 3b, e) and AG (Fig. 3c, f) orientations. Interestingly, S-HDAG enhanced the activities of TR-P1 (G) and (AG) more than TR-I5 (G) and (AG) promoters (Fig. 3b, c, e, f). Furthermore, TR-P1 (AG) was enhanced more than TR-P1 (G) by S-HDAG (Fig. 3b, c). In contrast, L-HDAG enhanced the activities of TR-I5 (AG) and (G) more than TR-P1 promoters (Fig. 3b, c, e, f). To further verify the participation of HDAGs, equal amounts of cellular lysate for reporter gene assay were analysed by Western blotting, as shown in Fig. 3(a, d). This is the first time that HDAGs have been shown to upregulate HDV endogenous cDNA promoters to differing degrees.

As L-HDAG has been shown to inhibit HDV replication (Chang et al., 1993; Chao et al., 1990; Modahl & Lai, 2000;
Wang & Lemon, 1993; Xia & Lai, 1992), we examined whether the coexistence of L- and S-HDAgs influences HDV cDNA promoter activities. A reporter gene assay was performed after cotransfection of equal amounts of plasmids that express HDAgs and plasmids that contain TR-P1 (G) or TR-P1 (AG) promoter sequence (Fig. 3g). Coexpression of L-HDAg had no apparent effect on S-HDAg activity. S-HDAg maintained the ability to upregulate TR-P1 promoters in the presence of L-HDAg in both G and AG orientations. Similar results were observed for TR-I5 (G) and (AG) promoter sequences (Fig. 3g). Our data suggested that the presence of L-HDAg has no influence on the upregulation of HDV endogenous cDNA promoters.

Fig. 3. Analysis of HDV cDNA promoter activity regulated by HDAgs. Luciferase activities of Huh7 cells cotransfected with various amounts of S-HDAg/pcDNA3.1 or L-HDAg/pcDNA3.1 plasmids and pGL-3 basic containing G (b) or AG (c) of TR-P1, and G (e) or AG (f) of TR-I5 HDV cDNA constructs. pcDNA3.1 was used to supplement DNA of each transfection to reach a final concentration of 10 µg. To examine the expressions of HDAgs in each transfection, equal amounts of lysates (30 µg) were analysed by Western blotting (a, d). To investigate the role of coexistence of HDAgs with HDV-specific promoters (g), S- and L-HDAg-expressing plasmids (3 µg) were cotransfected with TR-P1 or TR-I5 (4 µg). Luciferase activity was subsequently measured. *Significant difference when compared with control (Student’s t-test, P<0.01).
Mutations in TR-P1 sequences result in defective HDV RNA accumulation

S-HDAg is indispensable for HDV RNA replication (Kuo et al., 1989). Thus, we established an Huh7 cell line that stably expresses S-HDAg (Huh-S) to support HDV cDNA-free RNA replication. Using wild-type (WT) HDV 1.9 kb in vitro-transcribed RNA as the positive control, we further characterized the importance of TR-P1 and TR-I5 sequences for HDV de novo RNA synthesis (Fig. 4b, c). The WT and mutated HDV RNAs were transfected separately into Huh-S cells. Samples were collected at the indicated time points (0, 1, 2, 4 days) and the extracted RNAs were analysed by Northern blotting. The accumulations of mutated RNAs (right panel in Fig. 4b and c) were compared with corresponding WT controls (left panel in Fig. 4b and c) on the same gels.

As the first 20 nt sequence of ORF is essential for maximal TR-P1 promoter activity (Fig. 2a), the role of the 1601-AUG site in HDV RNA synthesis was analysed. The sequence of AUG was mutated to an amber stop codon UAG by site-directed mutagenesis (Fig. 4a, muAUG) and the genomic and antigenomic in vitro-transcribed HDV 1.9 kb RNAs were transfected separately into Huh-S cells. Following transfection, newly synthesized RNA was detected as indicated by the number of days post-transfection (Fig. 4b, c). On day 1 post-transfection of the WT genomic HDV 1.9 kb RNA, the antigenomic-sense HDV RNA monomer was detected. The HDV RNA dimer was detected on day 2 post-transfection (Fig. 4b, WT). When muAUG-HDV 1.9 kb genomic RNA was transfected, monomeric HDV RNA was detected on day 1 post-transfection. However, dimeric RNA was undetectable even on day 4 (Fig. 4b). The HDV genomic RNA products synthesized from WT antigenomic RNA transfection (Fig. 4c) were similar to those synthesized from antigenomic RNA (Fig. 4b) but with approximately 1 day delay. Although AUG mutation to UAG blocks the formation of HDAg ORF at the HDV genome and inhibits S-HDAg expression, the continuous expression of exogenous S-HDAg protein supports the integrity of HDV RNA replication. The data suggested that the AUG site of ORF plays an important role in driving the HDV double rolling-circle RNA replication and destruction of the ORF severely reduces HDV RNA replication.

To further examine the role that TR-P1 plays in HDV RNA synthesis, in vitro-transcribed 1.9 kb RNAs of P1-m5 and P1-m2 (Fig. 4a) were transfected into Huh-S cells. On day 4 post-transfection, neither antigenomic-sense (Fig. 4b) nor genomic-sense (Fig. 4c) RNA was present in cells transfected with P1-m5 RNA. Interestingly, when P1-m2 1.9 kb genomic RNA was transfected, fragments smaller than monomeric HDV RNA were detected (Fig. 4b). Surprisingly, the genomic-sense HDV RNA monomer was detected on day 4 post-transfection of antigenomic P1-m2 RNA (Fig. 4c). These results indicated that the effect of P1-m5 mutations was more overwhelming than that of P1-m2 mutations, as P1-m5 RNA constructs completely lost the ability to be replicated. This further supported the importance of the TR-P1 sequence in HDV RNA replication.

Mutations in TR-I5 sequences also result in defective HDV RNA accumulation

From the data in Fig. 2(b), the sequence located between nt 1314 and 1363 is important for promoter activity. Therefore, we compared HDV genomic sequences between nt 1314 and 1363 to analyse the consensus of TR-I5 sequences from ten isolates (Fig. 5a). Several consensus sequences were observed. To further investigate whether TR-I5 is involved in HDV RNA replication, mutated TR-I5 sequences that extend from I5-m2 (Figs 1 and 2b) were
employed at the CCGGC element (Fig. 5a, grey area, US, nt 1351–1355) and the sequences of I5-m1 to m5 constructs were confirmed by DNA autosequencing (Fig. 5b). In order to maintain the integrity of the ORF, the amino acid at position 83 of HDAg was changed from alanine to isoleucine (A83I) and that at position 84 was changed from glycine to leucine (G84L).

After the in vitro 1.9 kb genomic RNAs encoding I5-m1 to m5 mutations were purified, transfection experiments were carried out in Huh-S cells. The accumulations of mutated RNAs (right panels in Fig. 5c and d) were compared with corresponding WT controls (left panels in Fig. 5c and d) on the same gels. With the I5-m5 construct, synthesis of antigenomic-sense RNA was completely inhibited even on day 4 post-transfection (Fig. 5c). With the constructs of I5-m3 and I5-m2, synthesis of the monomeric RNA was delayed to day 2 post-transfection. On day 4 post-transfection, only monomeric RNA was detected (Fig. 5c). With only 1 nt mutated at I5-m1, monomeric RNA was detected on day 1 post-transfection, but dimeric RNA was not. RNA fragments smaller than a dimer were

Fig. 5. Detection of HDV RNA accumulation in Huh-S cells transfected with in vitro-transcribed HDV 1.9 kb RNA containing mutated TR-I5 elements. (a) Sequence comparison of nt 1314–1363 in TR-I5 with ten isolated HDV genomes. (b) Site-directed mutation of TR-I5 within 1.9 kb/pKS by DNA autosequencing. The region of mutation is shown (underline). (c, d) Northern blot hybridization analysis of total RNA (1 μg) extracted from Huh-S cells transfected with genomic or antigenomic WT and mutated in vitro-transcribed RNAs. Genomic- and antigenomic-sense HDV RNAs were detected with DIG-labelled strand-specific RNA probes. Total RNA (1 μg) from representative WT and I5-m5 stained with EtBr served as a loading control.
detected as a smear signal between monomeric and dimeric HDV RNA (Fig. 5c).

We also transfected antigenic 1.9 kb HDV I5 RNA mutants to detect the de novo-synthesized genomic-sense HDV RNA. In cells that were transfected with the I5-m5 RNA construct, the genomic-sense RNA was not detected on day 4 post-transfection (Fig. 5d). The synthesis of genomic-sense RNA dimer with I5-m3 and I5-m2 constructs was delayed to day 4 post-transfection (Fig. 5d). Interestingly, the replication ability of the I5-m1 antigenic RNA construct was not affected compared with WT RNA transfection (Fig. 5d). Our data showed that the efficiency of HDV RNA double rolling-circle replication is related to the specific site mutations and that antigenic RNA rolling-circle synthesis is affected more severely than genomic RNA synthesis by the I5-m1 mutation. These results suggested that the CCGGC element at the TR-I5 sequence is associated with HDV double rolling-circle replication.

Unit-length HDV RNA monomer is edited to express L-HDAg independently of double rolling-circle RNA synthesis

To determine whether the expressions of HDAgs are affected by mutated TR-P1 and TR-I5 sequences, *in vitro*-purified genomic and antigenomic 1.9 kb HDV RNAs were transfected into Huh-S cells, respectively. HDAgs were detected by Western blot analysis on day 4 post-transfection. The data showed that Huh-S cells express exogenous S-HDAg (Fig. 6a, b; N) and L-HDAg is synthesized in cells transfected with WT genomic (Fig. 6a, WT) or antigenic (Fig. 6b, WT) HDV RNA. L-HDAg was not detected with muAUG, P1-m5 or P1-m2 mutations (Fig. 6a, b). The absence of L-HDAg with the muAUG mutant resulted from the lack of an ORF of HDAg due to the AUG to UAG mutation. Although monomeric HDV RNA replication occurred in the muAUG mutant (Fig. 4b, c), neither S- nor L-HDAg protein was synthesized with the monomeric HDV RNA. P1-m2 and P1-m5 mutants may have caused inhibition of HDAg mRNA transcription, significantly affecting replication and inhibiting transcription. The I5-m5 mutated RNA constructs completely inhibited HDV RNA replication (Fig. 5c, d), resulting in the absence of detectable L-HDAg. Surprisingly, although only the monomeric HDV RNA was detected in the I5-m3 mutant (Fig. 5c, d), L-HDAg synthesis was not affected by the I5-m3 RNA mutant on day 4 post-transfection. Apparently, the editing event of the antigenic HDV RNA was preserved in the monomeric HDV RNA. The edited HDV RNAs caused the expression to switch from S-HDAg only to that of both HDAgs. As the genomic HDV RNA has to be synthesized first from the *in vitro*-synthesized 1.9 kb antigenomic RNA for future replication and further editing in new antigenomic RNA, the inefficiency and instability of the transfected antigenomic RNA may explain why there was less L-HDAg synthesized from antigenomic 1.9 kb RNA (Fig. 6b). In addition to the presence of L-HDAg, the level of S-HDAg increased in I5-m3 RNA mutants. These results suggested that transcription of S-HDAg mRNA also occurs in the I5-m3 RNA construct that is defective in dimeric HDV RNA synthesis. Therefore, HDV RNA editing is independent of RNA double rolling-circle replication. Although the I5-m2 and I5-m1 mutants possessed a defective HDV RNA dimer, both HDAgs were expressed at levels similar to that of WT HDV RNA (Fig. 6a). These results suggested that the CCGGC element at TR-I5 region is a site associated with HDV RNA double rolling-circle replication instead of a promoter of HDV replication or mRNA transcription.

DISCUSSION

The objective of this study was to investigate the *cis*-elements in the HDV genome that are critical for RNA-dependent RNA replication. We identified a novel cDNA region of HDV, denoted TR-I5, located within the HDAg ORF. Another promoter sequence was located in front of the HDAg ORF and was denoted TR-P1. Neither TR-P1 nor TR-I5 has been shown to be involved in ribozyme-activity regions required for HDV RNA replication (Jeng *et al.*, 1996). The promoter activity of TR-I5 was greater than that of TR-P1 in a luciferase reporter gene assay. The TR-P1 promoter region has been studied extensively and found to contain an HDV RNA–RNA replication promoter mediated by RNA polymerases (Abraham &
Pelchat, 2008; Beard et al., 1996; Chang et al., 2008; Greco-Stewart et al., 2007, 2009; Lehmann et al., 2007). Our results showed that the sequence located at nt 1602–1683 and the first 20 nt overlapping the N terminus of the ORF sequence (nt 1582–1601) are equally important for TR-P1 promoter activity. In addition, the sequence between nt 1582 and 1683 is critical for HDV cDNA promoter activity.

Using the linker-scanning mutagenesis method, a StuI site (nt 1335) important for HDV replication but dispensable for transcription of HDAg has been identified (Wang et al., 1997). Interestingly, this StuI site is located in the TR-I5 region (nt 1223–1363). When this StuI site was deleted to generate TR-I6, the promoter activity was reduced significantly. Furthermore, mutations in a highly conserved region between nt 1314 and 1363 (I5-m2) significantly reduced promoter activity. It is logically assumed that the TR-I5 sequence possesses a promoter element that is associated with HDV replication.

HDAG interactions with HDV RNA have been reported. The RNA conformation is altered when HDAGs bind to HDV RNA (Huang & Wu, 1998) and HDAGs stimulate in vitro elongation by host RNA pol II (Yamaguchi et al., 2001). These findings indicate the essential role of HDAGs in HDV RNA replication and transcription. Although transactivation of heterologous promoter expression by L-HDAG has been reported (Goto et al., 2000; Wei & Ganem, 1998), it is unclear how HDAGs regulate their own HDV promoters. Our data showed that S-HDAG has the strongest stimulating effect on TR-P1, especially in the antigenomic sense. It is possible that S-HDAG regulates its own promoter at the beginning of infection to initiate mRNA synthesis. Interestingly, L-HDAG stimulates TR-I5 promoter activity to a higher level than that of TR-P1. Although L-HDAG inhibition of genomic RNA synthesis has been reported (Modahl & Lai, 2000), coexistence of both L- and S-HDAGs neither inhibits nor enhances S-HDAG-induced stimulation of TR-P1 and TR-I5 promoter activities (Fig. 3g). These results suggested that there is no mutual influence of L- and S-HDAGs, at least on the pol II-mediated HDV-specific reporter assay. TR-P1 and TR-I5 contain cDNA promoter activities and their activities can be specifically regulated by HDAGs.

We further investigated whether HDAGs synthesized from the ORF of the HDV genome are required for HDV RNA replication. Both genomic and antigenomic HDV monomeric RNAs were synthesized (Fig. 4, muAUG) without HDV dimeric RNA even at 8 days post-transfection (data not shown). The data suggested that stably expressed exogenous S-HDAG is enough to initiate HDV RNA synthesis but loss of the AUG start codon results in defective RNA replication. In host cells, HDV RNAs are synthesized by cellular RNA polymerases. However, the intrinsically high error rate of these enzymes is a major contributor to the generation of extreme population diversity that facilitates virus adaptation and evolution. Sequence analysis of HDV shows an evolution rate of 3.0 × 10⁻²–3.0 × 10⁻³ substitutions nt⁻¹ year⁻¹ (Chao et al., 1994; Krushkal & Li, 1995; Lee et al., 1992). This high mutation rate for RNA synthesis causes HDV quasispecies to exist in host cells. Thus, we propose that the AUG site of the ORF in HDV acts as a recognition site to ensure that HDV RNA descendants express S-HDAG and proliferate to the next infected cell. If recognition site(s) are absent, such as in the AUG mutation, RNA synthesis via the double rolling-circle mechanism stops, regardless of whether initiation has occurred.

It has been demonstrated that the TR-P1 region acts as an RNA promoter for transcription (Beard et al., 1996). Surprisingly, with the P1-m2 1.9 kb RNA mutant, RNA fragments smaller than monomeric HDV RNA were detected (Fig. 4b), and the genomic-sense RNA monomer was detected on day 4 post-transfection (Fig. 4c). The data suggested that the P1-m2 mutant loses replication ability, although not completely, as the specific polymerases are able to carry out full-length HDV RNA synthesis with P1-m2 mutations, but to varying degrees. As pol I is considered responsible for antigenomic HDV RNA synthesis (Lai, 2005), it is possible that, in the presence of P1-m2 mutations, pol I initiates antigenomic RNA synthesis but does not successfully synthesize monomeric HDV RNA. In contrast, pol II can synthesize the genomic HDV RNA monomer (Lai, 2005) with the P1-m2 mutant. As newly synthesized S-HDAG was not observed adding to the exogenous S-HDAG, it is possible that this genomic HDV RNA monomer cannot be used for mRNA transcription (Fig. 6b, P1-m2).

The role of the CCGGC element in the regulation of HDV RNA double rolling-circle replication is a novel finding. Mutations of all 5 nt to AAAAU completely inhibited HDV replication. In the presence of 1–3 nt mutations at this element, HDV RNA double rolling-circle replication was hampered to differing degrees. As TR-I5 contains cDNA promoter activity, we speculated that recognition of this element by the polymerase is required for replication to occur. It is possible that the CCGGC element of HDV RNA acts as a second recognition site to ensure the accuracy of HDV full-length RNA synthesis.

Interestingly, both S- and L-HDAGs were expressed normally in cells transfected with genomic and antigenomic I5-m3 RNAs on day 4 post-transfection (Fig. 6). In contrast, only the HDV RNA monomers were detected under the same conditions (Fig. 5). Unfortunately, HDAG mRNA was not detected by DIG-labelled Northern blot analysis in this study. However, the results provided evidence that an HDV RNA-editing event occurs in the HDV RNA monomer independent of double rolling-circle replication. Furthermore, both S- and L-HDAG mRNA transcriptions are independent of double rolling-circle replication. We conclude that the TR-P1 region is probably the only authentic HDV RNA promoter for mRNA, genomic and antigenomic RNA synthesis. Although TR-I5 exhibited greater promoter activity than TR-P1 in the
luciferase reporter gene assay, it is not an RNA promoter but rather is associated with RNA double rolling-circle replication. The CCGGC element of the TR-I5 region is important for HDV replication but not for transcription of HDAGs. The integrity of the HDAg ORF is essential for HDV RNA double rolling-circle replication to synthesize the HDV RNA dimer but not the HDV RNA monomer.

**METHODS**

**Cell culture.** A human hepatoma cell line (Huh7) (Nakabayashi et al., 1982) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) containing 10 % FBS (HyClone), 2 mM L-glutamine, 100 IU penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (Gibco) and maintained at 37 °C in a 5 % CO₂ humidified atmosphere. The Huh7 cell line that stably expresses S-HDAg, Huh-S, was cultured under the same conditions but with the addition of 0.4 mg G418 ml⁻¹ (Sigma-Aldrich) to the medium.

**Construction of HDV cDNA sequence for luciferase reporter gene assay.** The 1.9 kb/pKS sequence was used to design primers in the corresponding location as described previously (Jeng et al., 1996). The cDNA fragments were PCR-amplified by a designated primer set (Supplementary Table S1, available in JGV Online) and cloned into TA vector (Yeastern Biotech). HDV cDNA fragments were recloned into pG3-3 basic plasmid (Promega) with Kpnl and BglII restriction enzyme sites in both orientations (Supplementary Fig. S1). DNA sequences of all constructs were verified by DNA autosequencing.

**Site-directed mutagenesis of HDV cDNA.** The sequences of muAUG, P1-m5, P1-m2, I5-m5, I5-m3, I5-m2 and I5-m1 were derived from WT HDV 1.9 kb/pKS plasmid DNA by using a QuikChange Site-Directed Mutagenesis kit (Agilent Technologies) according to the manufacturer’s instructions. The mutagenic primers used in site-directed mutation are described in Supplementary Table S1.

**In vitro transcription of HDV RNA.** The method for in vitro transcription of HDV RNA has been described previously (Sheu, 2002). Briefly, HDV genomic 1.9 kb RNAs were transcribed from WT and mutated HDV 1.9 kb/pKS plasmids with AmpliScribe T7 High 2002. Briefly, HDV genomic 1.9 kb RNAs were transcribed from WT and mutated HDV 1.9 kb/pKS plasmids with AmpliScribe T7 High 2002. (Promega) after linearization by BglII digestion. HDV antigenomic RNAs were transcribed from the same plasmids using AmpliScribe SP6 Transcription kit (Epicentre) after linearization by SmbI digestion. To prevent cDNA contamination, synthesized RNAs were treated with DNase (Epicentre) followed by RNA extraction.

**Luciferase reporter gene assay.** Cells were seeded onto 60 mm dishes in duplicate and incubated at 37 °C for 18 h until the density reached 70 % confluence. The efficiency of transfection was determined by cotransfection with pSV-β-Gal (Promega), assay of β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside (ONPG; Promega) and measurement of A420. Transfections were performed with Transfast reagent (Promega). The reporter plasmid of pGL-3 basic (Promega) containing the HDV cDNA sequence (4 µg) was mixed with pSV-β-Gal (1 µg) in Transfast (15 µl) in serum-free DMEM (2 ml). The DNA/reagent mixture was then added directly to the Huh7 cells followed by incubation at 37 °C for 48 h. Next, cells were harvested and lysed with cell culture lysis reagent (Promega). Equal amounts of cell lysates (20 µl) were used to measure enzyme activities with a Microplate Luminometer (TR717; Applied Biosystems). After calibration of transfection efficiency using β-galactosidase activity, the fold difference in transcription activity was determined. Each value represents the mean of a minimum of three independent transfections after normalization to cotransfected β-galactosidase activities.

**Cotransfection of HDAGs with reporter plasmid.** Huh7 cells were transiently cotransfected with constructed reporter plasmid (4 µg), pSV-β-Gal (1 µg) and expression plasmids of S-HDAg and L-HDAg (1–5 µg) were mixed with Transfast (DNA : reagent = 1 : 3) in serum-free DMEM (2 ml) on 60 mm dishes. After 48 h incubation, the cells were harvested for reporter assay. Successful transfection was verified by Western blot analysis.

**Transfection of in vitro-synthesized RNA.** Transfection was performed with Lipofectamine 2000 (Invitrogen) as described previously (Liao et al., 2009). Briefly, Huh-S cells were seeded onto a 60 mm dish with 50 % confluence and incubated for 24 h. Lipofectamine 2000 (12 µl) was diluted into 0.5 ml opti-MEM (Gibco) and incubated at room temperature for 5 min. In vitro-transcribed RNA (4 µg) was diluted into another 0.5 ml opti-MEM. The diluted RNA was then combined with diluted Lipofectamine 2000 and incubated at room temperature for 20 min. After the culture medium was washed with PBS and replaced with 1 ml opti-MEM, the RNA–liposome complexes were added to Huh-S cells. After 3 h incubation, the medium was replaced with fresh 10 % FBS/DMEM.

**Northern blot hybridization analysis.** Total RNA was extracted from Huh-S cells transfected with in vitro RNA using TriPure Isolation Reagent (Roche). One microgram of RNA was electrophoresed through an agarose (1.2 %)/MOPS gel containing 2 % formaldehyde and blotted onto a positively charged nylon membrane (Pall Corporation). Detection of HDV RNA was performed with DIG-labelled strand-specific RNA probes using a DIG Northern Starter kit (Roche) according to the manufacturer’s protocol. In brief, to synthesize the genomic-sense RNA probe or antigenic-sense RNA probe, S29 or S18 plasmids (Modahl & Lai, 1998) were linearized with HindIII and transfected with T7 RNA polymerase (Roche). Hybridization, at 68 °C for 16 h, was followed by blocking of the membrane and incubation in anti-DIG–alkaline phosphatase solution (1 : 10 000) for 30 min. Membranes were then washed twice in DIG washing buffer for 15 min and once in DIG detection buffer for 5 min. Imaging was carried out with CDP-Star ready-to-use solution (Roche) and X-ray film (Fujiﬁlm) for 1–5 min.

**Western blot analysis.** Cells were washed with PBS and harvested with RIPA buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS, 2 mM EDTA, 1 mM PMSF) in the presence of Protease Inhibitor Cocktail (Roche). The protein concentration was determined by Bradford assay (Bio-Rad). Thirty micrograms of cell lysates was separated by SDS-PAGE (12.5 % polyacrylamide). After transfer to PVDF membrane (Pall), the proteins were reacted with polyclonal anti-HDAg or anti-β-actin (Sigma-Aldrich) antibody, followed by anti-rabbit or anti-mouse IgG conjugated to HRP (Zymed). A chemiluminescence detection kit (ECL; Millipore) was applied to determine the levels of protein expression.

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**REFERENCES**


