Initiation of hepatitis delta virus (HDV) replication: HDV RNA encoding the large delta antigen cannot replicate

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The hepatitis delta virus (HDV) nucleocapsid consists of a genomic-length RNA of 1-7 kb and approximately equimolar amounts of the small and large forms of the hepatitis delta antigen (S-HDAg and L-HDAg, respectively). Since HDV RNA particles contain not only a genomic RNA species encoding S-HDAg but also an RNA species encoding L-HDAg, which is produced by an RNA-editing process, the question arises as to whether RNAs encoding either L-HDAg or S-HDAg can initiate replication. To study this, two cDNA-free transfection methods were employed: HDV RNA cotransfected with either the S-HDAg-encoding mRNA species or the ribonucleocapsid protein complex, comprising HDV RNA and recombinant S-HDAg. Results showed that the genomic-sense RNA encoding S-HDAg could promote HDV replication, whereas the L-HDAg-encoding RNA species was unable to replicate under the same conditions. The antigenomic RNA species encoding either S-HDAg or L-HDAg could not replicate by either of these procedures. In addition, L-HDAg alone could not promote replication of the genomic RNA but, by supplementing an equal amount of S-HDAg, replication occurred. These data indicate that the genomic RNA species encoding S-HDAg is the only functional genome for HDV infection and explain why the presence of the edited HDV RNA encoding L-HDAg does not interfere with HDV infection.

Introduction

Hepatitis delta virus (HDV) is a subvirus particle that requires hepatitis B virus (HBV) envelope protein for forming infectious virus particles (Rizzetto et al., 1980). HDV particles contain a nucleocapsid that consists of a 1-7 kb RNA genome and almost equal amounts of the small (195 aa) and large (214 aa) forms of the hepatitis delta antigen (S-HDAg and L-HDAg, respectively). HDV uses host cellular enzymes to synthesize its RNA genome (including both the genomic and antigenomic RNA species) independently from HBV (Lai, 1995). In HDV-infected cells, replication of HDV RNA is carried out via RNA-dependent RNA synthesis by an unknown mechanism. The DNA-dependent RNA polymerase II has been demonstrated to be responsible for the synthesis of a 0.8 kb mRNA (Modahl et al., 2000) but the polymerases mediating the synthesis of HDV antigenomic and genomic RNA have not been identified.

Current evidence suggests that antigenomic RNA synthesis is mediated by an enzyme other than pol II (Modahl et al., 2000). With the addition of polymerase inhibitors, Moraleda & Taylor (2001) have shown that HDAg mRNA and genomic HDV RNA synthesis were inhibited by α-amanitin. Therefore, pol II, or a pol II-like enzyme, is mediating HDV replication.

The essential role of S-HDAg in HDV replication has been well established by several different experimental approaches (Lai, 1995). In a cDNA transfection experiment, the disruption of the S-HDAg-encoding sequence by a 2 nt deletion resulted in the loss of RNA replication. However, S-HDAg supplied in trans could restore replication (Kuo et al., 1989). Similar findings were obtained by transfection of the ribonucleocapsid protein (RNP) complex (using HDV RNA plus a recombinant form of HDAg derived from Escherichia coli), in which blocking the open reading frame (ORF) of S-HDAg in the viral genome disabled RNA replication (Dingle et al., 1998). Cotransfection of a plasmid encoding a modified form of S-HDAg with a histidine tag (His-tag) could not restore the replication of HDV cDNA encoding defective S-HDAg (Dingle et al., 1998). These...
data indicate that intact S-HDAg is required for HDV replication. However, transfection using RNP made from recombinant S-HDAg modified with His-tag at either terminus, together with the genomic RNA encoding intact S-HDAg, could lead to RNA replication (Dingle et al., 1998). One interpretation of these findings is that the modified S-HDAg could carry out only a certain aspect of the HDV replication cycle, such as the transfer of the RNP complex to the nucleus, but that initiation of RNA replication may require newly synthesized S-HDAg that is properly post-translationally modified. These observations suggest that the structural requirement of HDV RNA for replication at the initiation stage may differ from that for the maintenance stage in the HDV life cycle.

The HDV virion contains both L-HDAg and S-HDAg, together with genomic RNA in an RNP complex (Bergmann & Gerin, 1986; Bonino et al., 1986). Once entering the nucleus of cells, HDV RNP is thought to initiate replication by a rolling-circle process, which leads to the synthesis of antigenic RNA and S-HDAg-encoding mRNA (Lai, 1995). Synthesis of genomic RNA from the newly synthesized antigenic RNA is, presumably, delayed until a sufficient amount of S-HDAg is synthesized from the newly transcribed mRNA in order to reverse the inhibitory effects of L-HDAg (Modahl & Lai, 2000). During RNA replication, an RNA-editing process occurs, resulting in the production of a mRNA encoding L-HDAg (Casey & Gerin, 1995), which will shut off further RNA replication and initiate virus assembly.

The RNA-editing process takes place on the antigenic RNA (Casey & Gerin, 1995), producing an RNA that has an ORF encoding L-HDAg. This RNA is then used as the template for the synthesis of its genomic counterpart. The edited genomic RNA encoding L-HDAg is used as the template to produce L-HDAg mRNA.

Accordingly, during the HDV life cycle, there are several genomic-length RNA species produced: (1) genomic RNA encoding S-HDAg; (2) genomic RNA encoding L-HDAg; (3) antigenic RNA encoding S-HDAg; and (4) antigenic RNA encoding L-HDAg. Since HDAg can bind both genomic and antigenic RNAs equally well (Lin et al., 1990; Hwang et al., 1992), all four of these RNA species are expected to be associated with S-HDAg and L-HDAg proteins to form RNP complexes. If all four of these RNA species exist in the infected cell, what are their functions and destiny during the virus life cycle? Although both genomic and antigenic RNAs are present in the nucleus of the infected cell, only the genomic-sense RNAs (RNA species 1 and 2) are found in the virus particle (Chang et al., 1991; Ryu et al., 1993). The mechanism for the preferential selection of the genomic HDV RNA species for packaging into virus particles is still unknown. We postulate that an additional step of selection for genomic and antigenic RNA might occur at the initiation stage of replication as well. Therefore, in this study, we set out to examine the ability of the various HDV RNA species to replicate in the presence of recombinant S-HDAg or mRNA encoding either L-HDAg or S-HDAg to understand the requirements for initiation of HDV replication at the early stage of virus infection.

We demonstrate that the nucleocapsid that comprises the L-HDAg-encoding genomic RNA species, which is generated by RNA editing, is unable to initiate replication even in the absence of S-HDAg. L-HDAg does not support HDV RNA replication nor does it inhibit the initiation of RNA replication. These findings explain why HDV particles, which contain S-HDAg and L-HDAg and a genomic RNA encoding S-HDAg, are able to initiate HDV RNA replication.

Methods

- **Preparation of recombinant S-HDAg.** Preparation of recombinant S-HDAg from E. coli followed the procedures described previously by Sheu & Lai (2000).

- **Site-directed mutagenesis of HDV cDNA.** The 1.9 kb plasmid of HDV cDNA encoding the S-HDAg ORF (S-Ag/1.9) was mutated to encode the L-HDAg ORF (L-Ag/1.9) by site-directed mutagenesis. The termination codon (TAG) in the S-HDAg-encoding sequence was changed to TGG. Three primers were used for introducing this mutation. The 1.4 kb PCR product was used as a template for introducing the TGG mutation primer (5′ AGCCAGGGATTCCCATTTGATA) to amplify a 144 bp mega-primer. This mega-primer was used for second-round PCR using a 2.4 kb primer (5′ GTCACCACTCTTAAGCTTCTCT) containing an AflI site (italics). The S-Ag/1.9 fragment was subcloned into the 1.9 kb HDV cDNA to replace the same fragment that encodes S-HDAg. The L-HDAg-encoding clone (L-Ag/1.9) was verified by DNA sequencing using Sequenase 2.0 (US Biochemical).

- **In vitro transcription of HDV RNA.** Genomic HDV RNA (1.9 kb) was transcribed from plasmids S-Ag/1.9 and L-Ag/1.9 using T7 MEGAscript (Ambion) after linearization by EcoRV digestion. Antigenic HDV RNA was transcribed from S-Ag/1.9 and L-Ag/1.9 using SP6 MEGAscript (Ambion) after linearization by SmaI digestion. The mRNAs of S-HDAg and L-HDAg were prepared from the expression plasmids (Sheu & Lai, 2000), which had been linearized with BamHI and synthesized with T7 mMESSAGE mMACHINE (Ambion).

- **Cell culture.** COS-7 cells were cultured at 37°C in 35 mm diameter dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% foetal bovine serum (FBS). 100 IU penicillin/ml and 100 µg streptomycin/ml. TS63 cells, which contain an integrated cDNA with an HDV-encoding ORF (Hwang et al., 1995), were grown at 33°C and 5% CO₂ in DMEM supplemented with 10% FBS, 800 µg/ml G418 and Penicillin–Streptomycin.

- **Transfection.** Purified recombinant S-HDAg (1 µl, 0.4 µg) or mRNA encoding either L-HDAg or S-HDAg (1 µl, 1 µg) and HDV 1.9 kb RNA (3 µl, 3 µg) were mixed in a final volume of 25 µl with 10 mM HEPES buffer (pH 7.4) at room temperature for 10 min. DOTAP (15 µl, Boehringer Mannheim) and 10 mM HEPES buffer (pH 7.4, 35 µl) were preincubated at room temperature for 15 min and added to the RNA–protein mixture for transfection. Cells grown to subconfluency in 35 mm dishes were transfected with 1 ml fresh medium and 75 µl DOTAP–RNP or -RNA complex. In some experiments, in lieu of the RNP complex, L-Ag/1.9 or S-Ag/1.9 (3 µg) plasmid DNA was transfected under the same conditions as described above.
**Northern blot analysis.** Cellular RNA was extracted from transfected cells using TRIzol reagent (Gibco BRL) and 12 µg RNA was electrophoresed through a 1:2:2 agarose gel containing formaldehyde. RNA was blotted onto a nitrocellulose membrane (Hybond-C extra, Amersham) and probed with in vitro-transcribed HDV genomic RNA labelled with [32P]UTP to detect antigenomic RNA synthesis. To synthesize the genomic-sense RNA probe, the S29 plasmid (Modahl & Lai, 1998) was linearized with EcoRV and transcribed with T7 RNA polymerase. The membrane was prehybridized at 55 °C for 2 h in prehybridization buffer (0.9 M NaCl, 50 mM NaH2PO4, 5 mM EDTA, 0.5% SDS, 10 × Denhardt’s solution, 50% formamide, 400 µg/ml salmon sperm DNA and 100 µg/ml yeast tRNA) and hybridized overnight with hybridization buffer (0.63 M NaCl, 35 mM NaH2PO4, 3 mM EDTA, 0.5% SDS, 10 × Denhardt’s solution, 50% formamide and 100 µg/ml yeast tRNA) containing 2 × 106 c.p.m./ml 32P-labelled HDV genomic-sense RNA probe. To wash the membrane, washing buffer (1 × SSC and 0.5% SDS) was used for the low-stringency wash at 55 °C until the wash contained nearly background levels of radioactivity. The high-stringency wash was then performed with buffer (0.2% SSC and 0.5% SDS) at 80 °C for 30 min, followed by autoradiography.

**Western blot analysis.** Transfected cells were incubated with 150 µl RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl and 50 mM Tris–HCl, pH 8.0) in the presence of protease inhibitor cocktail (Boehringer Mannheim) at 4 °C for 10 min. The cell lysate was pipetted several times and 30 µl SDS-loading buffer was added. After the protein mixture was boiled for 10 min, 100 µl of sample was separated on a 10% polyacrylamide gel containing 0.1% SDS, 5% Glycerol, 0.1% bromophenol blue, 0.1% Sudan black B and 8 M urea. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R, destained, and dried. The blots were probed with anti-HDAg mAbs. Lanes: 1, 2, COS-7 cells transfected with recombinant S-HDAg; 3, 4, COS-7 cells transfected with recombinant S-HDAg and in vitro-synthesized 1.9 kb RNA of either genomic or antigenomic HDV RNA; 5, 6, COS-7 cells transfected with S-HDAg plus in vitro-synthesized dimeric HDV RNA of either genomic or antigenomic HDV RNA; 7, mock-transfected cells; and 8, COS-7 cells transfected with a trimeric cDNA of HDV as a positive control.

**Results**

**Genomic, but not antigenomic, RNA encoding S-HDAg can initiate RNA replication following RNP transfection.**

We first employed the RNP transfection method using recombinant S-HDAg and in vitro-transcribed HDV 1.9 kb RNA to determine the requirement for the initiation of HDV RNA replication. This method closely mimics natural virus infection. We used Western blot analysis of HDV RNA as an indication of HDV replication. Recombinant S-HDAg transfected into COS-7 cells in the absence of HDV RNA could still be detected at 3 days post-transfection (Fig. 1, lane 1) but disappeared by day 6 (Fig. 1, lane 2). When the genomic 1.9 kb RNA was included in the transfection, the amount of S-HDAg at day 6 was more than the amount of the transfected S-HDAg seen on day 3 (Fig. 1, lane 3). Furthermore, L-HDAg could be detected in this sample, indicating that HDV RNA replication had occurred and resulted in RNA editing. Only genomic-sense HDV RNA in combination with recombinant S-HDAg could promote HDV RNA replication (Fig. 1, lane 3), whereas under the same conditions, antigenomic-sense HDV RNA could not promote replication (Fig. 1, lane 4). We have also used dimeric HDV RNA instead of the 1.9 kb RNA in the RNP transfection assays. Again, using only the genomic-sense RNA, but not the antigenomic-sense RNA, led to replication (Fig. 1, lanes 5 and 6).

**Site-directed mutagenesis of HDV cDNA**

We next examined the replicating ability of the genomic RNA containing the L-HDAg-encoding ORF, which is generated following RNA editing and is packaged into the HDV virion (Xia et al., 1990). For this purpose, we used the 1.9 kb HDV cDNA containing the S-HDAg ORF (S-Ag/1.9) for site-specific mutation to change the ORF from S-HDAg to L-HDAg (L-Ag/1.9). The presence of the mutation was confirmed by DNA sequencing, which showed that residue A (corresponding to the termination codon for S-HDAg) was changed to a G residue, resulting in an extension of 19 aa in the ORF on the antigenic strand (Fig. 2). No other mutations were introduced. This L-Ag/1.9 cDNA clone was used for in vitro synthesis of the genomic 1.9 kb RNA containing the L-
HDAG ORF to be used for RNP transfection. Therefore, the RNA encoding L-HDAG differs by only 1 nt from the RNA encoding S-HDAG.

**L-HDAG-encoding genomic RNA cannot replicate in the cDNA-free RNA or RNP transfection experiments**

We next carried out a transfection experiment using the genomic RNA encoding L-HDAG together with either recombinant S-HDAG or S-HDAG mRNA in order to examine its ability to replicate. RNA replication was assessed by the detection of the antigenomic monomer (1–7 kb RNA) using Northern blot analysis. The results showed that the genomic 1–9 kb RNA encoding L-HDAG could not replicate either with the capped mRNA of S-HDAG (Fig. 3a, lane 3) or with recombinant S-HDAG (Fig. 3a, lane 4). Under the same conditions, HDV RNA replication occurred when the 1–9 kb RNA encoding S-HDAG was used for RNA or RNP transfection (Fig. 3a, lanes 1 and 2).

**L-HDAG alone cannot initiate HDV replication but replication occurs with the addition of S-HDAG**

L-HDAG has been shown to be a dominant–negative inhibitor of HDV RNA replication (Chao et al., 1990). Therefore, the above finding that the L-HDAG-encoding HDV genomic RNA could not replicate could have been due to the production of L-HDAG, which, in turn, inhibited subsequent RNA replication. However, the reported inhibitory effects of L-HDAG were based mainly on the cDNA transfection methods. Since DNA-dependent transcription itself is subjected to inhibition by L-HDAG (Lo et al., 1998), the inhibition of HDV RNA replication by L-HDAG observed could have been an indirect result of inhibition of RNA transcription from the transfected cDNA. When an RNA transfection method was used, inhibition by L-HDAG was observed mainly on genomic, but not antigenomic, RNA synthesis (Modahl & Lai, 2000). Furthermore, when recombinant L-HDAG was used, L-HDAG was able to support genomic RNA replication, probably as a result of the processing of L-HDAG into S-HDAG (Sheu & Lai, 2000). Thus, L-HDAG may not inhibit HDV RNA replication in natural HDV infection. Therefore, we examined further the role of L-HDAG in HDV infection.

We employed the RNA transfection method using the mRNA of L-HDAG to supply L-HDAG in trans in the cells. The capped mRNA of L-HDAG was synthesized in vitro and cotransfected with genomic-sense HDV RNA encoding S-HDAG or L-HDAG. RNA replication was monitored by Northern blot analysis of the antigenomic RNA or immunoblot analysis of HDAG. The result showed that L-HDAG synthesized from the transfected L-HDAG mRNA (Fig. 3b, lane 1) could be detected at 5 days post-transfection. However, no S-HDAG was detected, indicating that no RNA replication had occurred. Similarly, Northern blot analysis showed that HDV RNA replication did not occur when L-HDAG was cotransfected with either RNA encoding S-HDAG (Fig. 3a, lane 5) or RNA encoding L-HDAG (Fig. 3a, lane 6). However, when recombinant S-HDAG (Fig. 3a, lane 7) or its mRNA (Fig. 3a, lane 8) were used together with the mRNA encoding L-HDAG for transfection, RNA replication could be restored for the genomic RNA encoding S-HDAG, even when an equal amount of L-HDAG was present. These results confirmed the previous finding (Modahl & Lai, 2000) that L-HDAG did not significantly inhibit the synthesis of antigenomic RNA. Combined, these results suggest that the genomic RNA encoding L-HDAG is inherently defective for replication and that failure to replicate was not due to the production of L-HDAG.
Initiation of HDV replication

A control experiment using the cDNA transfection method for both S-Ag/1.9 and L-Ag/1.9 showed that cDNA encoding L-HDAg could not replicate (Fig. 3b, lane 4), whereas its counterpart cDNA encoding S-HDAg was able to replicate, as indicated by the production of both HDAg proteins (Fig. 3b, lane 2).

Antigenomic HDV RNA encoding L-HDAg cannot be used as a template for replication

The results above showed that the genomic RNA containing the L-HDAg ORF could not replicate; thus, it will be a dead-end product of HDV replication. We examined further the possibility that the antigenomic RNA encoding L-HDAg may be used as a template for replication, i.e. that RNA editing occurs on the antigenomic RNAs (Casey & Gerin, 1995); HDAg can complex with both the genomic and antigenomic RNA (Lin et al., 1990). We used recombinant S-HDAg to cotransfect with these RNA species. The results showed that neither the antigenomic RNA (Fig. 4, lane 2) nor the genomic RNA encoding L-HDAg (Fig. 4, lane 1) could initiate replication by the RNP transfection method. As a control, HDV RNA replication was detected from genomic, but not antigenomic, RNA encoding S-HDAg (Fig. 4, lane 4), which is in agreement with previous findings (Sheu & Lai, 2000).

L-HDAg-encoding HDV RNA cannot replicate in the S-HDAg-expressing TSδ3 cell line

To examine whether the inability of the HDV RNA encoding the L-HDAg ORF to replicate was due to the limitation of the amount of S-HDAg in the cell, in vitro-transcribed RNA encoding L-HDAg was transfected with or without S-HDAg into TSδ3 cells, which constitutively express S-HDAg from an integrated HDAg cDNA (Hwang et al., 1995). Transfection of genomic RNA encoding the S-HDAg ORF was capable of replicating with or even without help from exogenous S-HDAg (Fig. 5, lanes 1–3), indicating that S-HDAg produced in this cell line is capable of supporting HDV RNA replication. Again, the inclusion of L-HDAg-encoding mRNA in the transfection did not inhibit HDV RNA replication (Fig. 5, lane 4). In contrast, the genomic RNA encoding the L-HDAg ORF cotransfected with either S-HDAg mRNA (Fig. 5, lane 5) or recombinant S-HDAg (Fig. 5, lane 6) still could not replicate in the S-HDAg-expressing cell line. As a control, transfection of the HDV cDNA plasmid encoding the L-HDAg ORF into TSδ3 cells also could not replicate (Fig. 5, lane 9), whereas replication occurred when the plasmid encoding the S-HDAg ORF was transfected (Fig. 5, lane 8). Combined, these results indicated clearly that the HDV RNA with a L-HDAg ORF is inherently defective for RNA replication.

Discussion

During natural infection, the nucleocapsid of HDV is transported, upon entering the cells, to the nucleus where RNA replication occurs (Gowans et al., 1988); nuclear translocation of HDV RNA is mediated by HDAg (Chou et al., 1998). Once within the nucleus, initiation of HDV replication may require de novo-synthesized, intact S-HDAg (Dingle et al., 1998). Our results showed further that among the various RNA species in the HDV virion and infected cells, only those containing the S-HDAg ORF can replicate. Failure of the RNA encoding L-HDAg, which is a viral RNA species generated from RNA editing, to replicate may not be due to inhibition by L-HDAg but rather to an intrinsic defect of the RNA. These findings suggested an answer for solving a puzzling conceptual dilemma during the early stage of HDV infection, namely, why
L-HDAg does not accumulate early in the virus replication cycle.

Previous results indicated that only genomic, but not antigenomic, HDV RNA encoding S-HDAg could initiate RNA replication from the RNP complex containing the recombinant S-HDAg (Sheu & Lai, 2000). These data are consistent with the notion that HDV antigenomic RNA is involved only in the maintenance of HDV replication but not for initiation of HDV replication at the early stage of virus infection. However, using the cDNA-free RNA transfection method with capped mRNA, both genomic and antigenomic RNAs are able to initiate replication (Modahl & Lai, 1998). A possible explanation for the difference observed between RNP transfection and mRNA cotransfection is the presence of de novo-synthesized S-HDAg from the transfected mRNA, whereas, in the transfected RNP complex, the genomic, but not the antigenomic, template can produce S-HDAg mRNA. Thus, the RNP transfection method seems to be the method of choice for examining the early events of HDV replication, as it reflects more closely the natural requirements of HDV replication. Although the antigenomic RNA template could not be replicated in our RNP transfection assay, Dingle et al. (1998) showed that this RNA species could be replicated with recombinant S-HDAg purified from E. coli after cotransfection. One possible explanation is that in our RNP system, the efficiency of replication is below the detectable range. Nevertheless, under our assay conditions, antigenomic HDV RNA has a much lower ability for replication when compared to the genomic RNA in vitro.

The HDV virion contains RNAs encoding S-HDAg and L-HDAg (Xia et al., 1990). If both of these RNA species can replicate, it would be expected that L-HDAg would accumulate rapidly in infection and inhibit RNA replication. In this study, we used both the capped mRNA of S-HDAg and purified recombinant S-HDAg to cotransfect with in vitro-synthesized genomic RNA containing the L-HDAg ORF (Fig. 3a); replication did not occur in either case. Failure of the L-HDAg-encoding HDV RNA to replicate could have been due to insufficient amounts of S-HDAg in the cell or the production of L-HDAg, which, in turn, inhibits HDV RNA replication. However, this was found not to be the case, as this RNA failed to replicate in the S-HDAg-expressing cell line TFα3, which produces an abundant amount of S-HDAg (Fig. 5). Also, in agreement with our previous data (Modahl & Lai, 2000), we showed that L-HDAg does not inhibit replication of genomic HDV RNA as long as S-HDAg is present. Thus, the most likely possibility is that the single nucleotide substitution, which disrupted an amber termination codon, is detrimental to RNA replication. It is possible that this mutation may cause conformational changes to the HDV RNA. Previously, studies have shown that single nucleotide mutations in HDV RNA can cause significant effects on replication or transcription of various HDV RNA species (Wang et al., 1997). The mechanism of replication inhibition with the genomic RNA encoding L-HDAg is not demonstrated clearly in the present study. It is possible that inhibition caused by L-HDAg in trans or by other cellular factors after the initiation of replication could be involved in vivo, thus terminating any further replication.

The inhibitory effect of replication by L-HDAg reported previously was based on the cDNA transfection method (Chao et al., 1990; Glenn & White, 1991). A recent report by Modahl & Lai (2000) and the current findings suggest that the cDNA transfection approach may not reflect the real biology of HDV infection. We showed that the essential factors to initiate replication are nucleocapsid-associated S-HDAg and genomic RNA containing an S-HDAg ORF. Apparently, L-HDAg has little effect on the synthesis of antigenomic RNA from the incoming genomic RNA, as long as S-HDAg is present. Although recombinant S-HDAg purified from E. coli could also initiate replication from genomic RNA, it could not do the same with antigenomic RNA under our assay conditions. Our results suggest that E. coli-derived S-HDAg may be sufficient for initiating the synthesis of HDAg mRNA. However, newly synthesized HDAg (perhaps with correct post-translational modification) may be required for HDV RNA genome replication. This interpretation could explain why recombinant S-HDAg failed to support antigenomic RNA replication (Sheu & Lai, 2000); antigenomic RNA cannot encode mRNA directly, whereas S-HDAg-encoding mRNA can support its replication (Modahl & Lai, 1998).

Our data also support further the model proposed by Polson et al. (1996) that, after RNA editing, the edited antigenomic RNA with the L-HDAg ORF is replicated into the genomic RNA encoding L-HDAg. This RNA is then transcribed into L-HDAg mRNA without replicating into new antigenomic RNA containing L-HDAg. Additional evidences also support this observation: previous data from Ryu et al. (1993) have reported that the copy number of genomic RNA is 10-fold higher than that of antigenomic RNA in infected cells. Furthermore, Luo et al. (1990) have shown that up to 41% of genomic HDV RNA was edited to produce the L-HDAg ORF in HDV-infected chimpanzees. Interestingly, they also showed that only 3 of 100 cDNA clones were found to contain the UGG codon from the antigenomic RNA of the HDV-transfected cells. The ratio of these four HDV RNA species, genomic S-HDAg; genomic L-HDAg; antigenomic S-HDAg; antigenomic L-HDAg, is estimated to be 59:41:9:7:0:3, respectively. Therefore, antigenomic RNA containing the L-HDAg ORF has the lowest copy number of all four RNA species. These data indicated that antigenomic RNA containing the L-HDAg ORF may be produced solely from the editing process rather than from the replicated genomic RNA containing the L-HDAg ORF.

In conclusion, we have shown the critical requirements for HDV to initiate replication and the effect of S-HDAg associated with nucleocapsid. Among the four different combinations of HDV nucleocapsid, only one form, probably that comprising genomic RNA encoding S-HDAg and com-
plexed with S-HDAg, can initiate HDV replication. Thus, this regulation may account for the successful replication of HDV RNA after the virus enters the cells.

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