行政院國家科學委員會補助專題研究計畫成果報告

探討細胞內 D 型肝炎病毒之專一性核酸聚合酶之組成

Identification and characterization of HDV-specific cellular RNA polymerase machineries

計畫類別：[x]個別型計畫　　□整合型計畫

計畫編號：NSC90－2320－B－040－029

執行期間：90 年 08 月 01 日至 91 年 07 月 31 日

計畫主持人：許國堂

共同主持人：助理教授

計畫參與人員：碩士研究生 賴宗伯 廖威超

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執行單位：　　中山醫學大學

中華民國 91 年 10 月 26 日

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一、中文摘要

人類的D型肝炎病毒(HDV)具有一個環狀單股的核酸(RNA)做為它的基因體。因此，使用其 RNA 為鑄模來做為合成新基因體之用。到目前為止，HDV 的基因體中，只有發現一個基因。這個基因的產物就是 D 型肝炎病毒的抗原(HDAg) (Lai, 1995)。比較 HDAg 和已經知道的核酸聚合酶的序列，無法找到相似的地方和功能，所以，D 型肝炎病毒之所以能夠在動物細胞內複製，一定使用到細胞攜帶的核酸聚合酶(RdRP)。曾經有文獻報告指出，核酸聚合酶的 pol II 可能是主要的核酸聚合酶用來複製 HDV，可是 pol II 只能使用 DNA 為鑄模，如何扭轉它的專一性，還未有任何合理的解釋 (Modahl et al, 2000)。由於缺乏適合的模式研究被感染的細胞，以及可靠的試管內定性，使得尋找這個 HDV 專一的核酸聚合酶無法有效的進展。D 型肝炎病毒是唯一的會感染人類而且只在染色體外複製的 RNA 病毒，而且 D 型肝炎病毒進入細胞之後的複製和 B 型肝炎病毒無關。我們知道細胞內未知的核酸複製組合可以複製 D 型肝炎病毒，但是其中所含有之成分以及它的運作機制並未清楚地被證明出來。

我們已經建立了一個使用 HDV 的 RNA/HDAg (RNP)transfection 的感染細胞模式，在這個模式中完全避免了可能成為鑄模之 HDV 的 DNA，因此適合使用於鑑定只用 RNA 為鑄模之核酸聚合酶的組成 (Sheu and Lai, 2000)。為了尋找這個神秘地 RdRP，決定使用細胞基因之精簡雜交之差異表達(Subtractive hybridization)來對照出被 RNP 誘導的基因和被 DNA 誘導的基因之不同。從這個方法中應該會找到與 HDV 的複製相關的轉錄因子，進而推衍出真正的 RdRP 的基因。D 型肝炎病毒的核酸聚合酶和 D 型肝炎的致病性具有重要關係，瞭解它的本質對於治療 D 型肝炎是有實質的幫助。如果能夠從動物細胞內鑑定出從未被承認過的 RdRP 將會對於生命科學帶來重大的沖擊。未來，除了確定這個神秘地 RdRP 的組合成員之外，對於為何只有 HDV 的 RNA 序列會被使用於 RdRP 的活動中也應該加以研究。

關鍵詞：

人類的 D 型肝炎病毒，核酸聚合酶，精簡雜交之差異表達。

Abstract

Human hepatitis delta virus (HDV) has a single-stranded circular RNA genome that replicates by RNA-directed RNA synthesis. The virus encodes only a single protein product, the hepatitis delta antigen (HDAg). The HDAg lacks sequence homology to known RNA polymerases, suggesting that the virus employs a cellular polymerase for HDV replication (Lai, 1995). It has been proposed that replication of HDV is mediated by RNA polymerase II, a cellular

DNA-directed RNA polymerase Modahl et al, 2000). Due to the lack of natural infection for *in vivo* model and a reliable *in vitro* transcription assay system, the hunt for the human cellular RNA-directed RNA polymerase (RdRP) is hampered. The central dogma of cell biology would be changed if the cellular RdRP could be identified from human cells.

We have developed an *in vitro* RNA/protein (RNP) transfection system that initiates HDV replication without DNA contamination (Sheu and Lai, 2000). This RNP transfection system is a cDNA-free system that avoids the possible cross-interaction with DNA directed polymerases. Therefore, we propose to use this RNP transfection system to establish a subtractive hybridization screening for the identification of cellular RdRP machineries from human cells. From this screening, it is likely to identify many transcription factors associated with the RdRP activity. Thus, this three-year research project will be focused on the characterization of the HDV-specific RdRP machineries in order to unmask the mystery of cellular RdRP observed in HDV viral life cycle. The RdRP activity is strongly associated with the pathogenesis of chronic HDV infection.

Keywords:
(HDV)，(RdRP)，(Subtractive hybridization)

二、緣由與目的

在本計劃中，我們希望能夠達到下列的主要目標：

1. 從人類細胞中鑑識出與 RdRP 相關的組合成員。
2. 建立起一個可靠地、具有 HDV 的 RdRP 專一性的試管內合成 RNA 之方法，以供分析。

我們使用試管內合成之 D 型肝炎病毒 RNA和純化的重組型 D 型肝炎病毒抗原來組成核酸和蛋白質複合體，把這個核酸和蛋白質複合體轉殖到細胞內，可以偵測到 D 型肝炎病毒開始複製。使用這個方法來檢查隱藏在人類細胞內之只用 RNA 為鑄模之核酸聚合酶，以及 D 型肝炎病毒和宿主之間的互相牽制或利用的機制，或許可以揭開長久以來存在人類和病毒之間的一個演化上的答案。

三、結果與討論

We have applied PCR-select cDNA subtraction kit (Clontech) to analyze the mRNA purified from the HDAg/RNA transfected Cos7 cells. The PCR-amplified cDNA subtraction library was ligated to TA cloning vector. Subsequently after transformation into E.coli cells, the colonies were examined by PCR-analysis to identify the size of cDNA insert. Some of the cDNA clones have been sequenced to identify their identity.

Several gene sequences were found from the subtraction cDNA library. These cDNA clones including HLA-DRa, indolethylamine N-methyltransferase (INMT), profilin 1 (PFN1), ribosomal protein L41, farnesyl diphosphate synthase (FSPS), small nuclear ribonucleoprotein D2 (SNRPD2), RER1, heme oxygenase 2, beta actin, tubulin alpha 6, protein phosphatase 1 (catalytic subunit, gamma, PPP1CC), ribosomal protein S9, protein phosphatase 2 (regulatory subunit, PPP2R3), purine-rich element binding protin B, ATPase (ATP2B1), liver alkaline phosphatase.

To investigate the possible overexpression of those genes in HDAg/RNA transfected cells, Northern blot analysis were used to recheck the difference of those gene expression during HDV replication. Unfortunately, due to the sensitivity of Northern analysis, we haven't recognized the differentially expressed gene. To overcome this problem, we like to repeat the subtraction library construction and if possible, applying the real-time PCR to identify the HDAg/RNA complex induced genes.

計畫成果自評

We have sequenced several subtractive cDNA clones and they could be categorized into (1) ribosomal proteins, (2) protein

phosphatases, (3) cytoskeleton proteins, (4) RNA/DNA binding proteins, (5) methyltransferases, (6) HLADRa. Further investigation is needed to clarify these genes interactions upon HDV replication.

We also reported that L-HDAg-encoding RNA species are probably not involved in the initiation of HDV RNA synthesis; instead, their main function may be to serve as template for producing L-HDAg, which regulates HDV RNA synthesis and virion assembly (Sheu, 2002).

參考文獻

1. Lai, M. M. C. (1995). The molecular biology of hepatitis delta virus. *Annu Rev Biochem* 64, 259-286.
2. Modahl, L. E, Macnaughton, T. B., Zhu, N., Johnson, D. L., and Lai, M. M. C. (2000) RNA-dependent replication and transcription of hepatitis delta virus RNA involve distinct cellular RNA polymerases. *Mol Cell Biol* 20: 6030-9.
3. Sheu, G.-T and Lai, M..M..C. (2000). Recombinant hepatitis delta antigen from *E. coli* promotes hepatitis delta virus RNA replication only from the genomic strand but not the antigenomic strand. *Virology*, 278, 578-586.
4. Sheu, G.-T (2002) Initiation of hepatitis delta virus (HDV) replication: HDV RNA encoding the large delta antigen cannot replicate. *J Gen Virol.* 83, 2507-2513.

Journal of General Virology (2002), **83**, 2507–2513. Printed in Great Britain

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 L-HDAg-encoding RNA species are probably not involved in the initiation of HDV RNA synthesis; instead, their main function may be to serve as template for producing L-HDAg, which regulates HDV RNA synthesis and virion assembly. These results suggest 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.

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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

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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 antigenomic RNA and S-HDAg-encoding mRNA (Lai, 1995). Synthesis of genomic RNA from the newly synthesized antigenomic 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 antigenomic 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) antigenomic RNA encoding S-HDAg; and (4) antigenomic RNA encoding L-HDAg. Since HDAg can bind both genomic and antigenomic 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 antigenomic 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 anti-genomic 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 presence 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. First-round PCR used an upstream primer (5′ CACTGGG*GTCGAC*AA-CTCTG) with a *SalI* site (italics) and a TGG mutation primer (5′ AGCCAGGGATTCCCA<u>TGG</u>GATA) to amplify a 144 bp mega-primer. This mega-primer was then used for second-round PCR using a downstream primer (5′ GTCAACCT*CTTAAG*TTCCTCT) containing an *AflII* site (italics). The *SalI–AflII* 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 *Eco*RV digestion. Antigenomic HDV RNA was transcribed from S-Ag/1.9 and L-Ag/1.9 using SP6 MEGAscript (Ambion) after linearization by *Sna*BI digestion. The mRNAs of S-HDAg and L-HDAg were prepared from the expression plasmids (Sheu & Lai, 2000), which had been linearized with *Bam*HI, 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. TS*δ*3 cells, which contain an integrated cDNA with an S-HDAg-encoding ORF (Hwang *et al.*, 1995), were grown at 33 °C and 5% CO_2 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% 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 [^{32}P]UTP to detect antigenomic RNA synthesis. To synthesize the genomic-sense RNA probe, the S29 plasmid (Modahl & Lai, 1998) was linearized with *Eco*RV and transcribed with T7 RNA polymerase. The membrane was prehybridized at 55 °C for 2 h in prehybridization buffer (0·90 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, 0·5% SDS, 10× Denhardts' 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 NaH_2PO_4, 3 mM EDTA, 0·5% SDS, 10× Denhardts' solution, 50 % formamide and 100 μg/ml yeast tRNA) containing 2×10^6 c.p.m./ml ^{32}P-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. Proteins on the polyacrylamide gel were transferred with a semi-dry transfer cell (Bio-Rad) at 2·5 mA/cm^2 for 20 min. HDAgs were detected using the ECL Western Blot Detection system (Amersham) with a combination of three anti-HDAg monoclonal antibodies (mAbs) (Hwang & Lai, 1993).

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 HDAg 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).

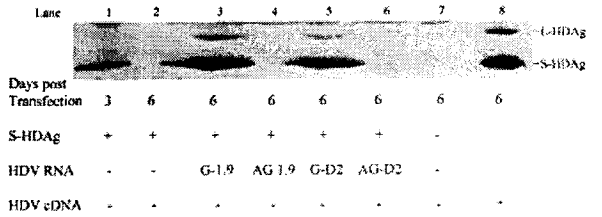

Fig. 1. HDV replication using the RNP transfection method, as detected by Western blot analysis of HDAg. Recombinant S-HDAg mixed with HDV RNA was transfected into COS-7 cells. Cell lysates were prepared at the indicated days post-transfection and used for immunoblot analysis using anti-HDAg mAbs. Lanes: 1, 2, COS-7 cells transfected with recombinant S-HDAg only; 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.

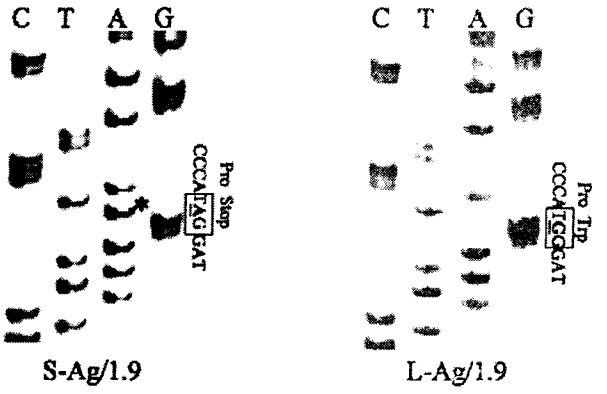

Fig. 2. Site-directed mutation of the S-HDAg ORF, which results in an L-HDAg ORF. The *SalI–AflII* fragment was sequenced and only the region of the mutation is shown.

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 antigenomic 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-

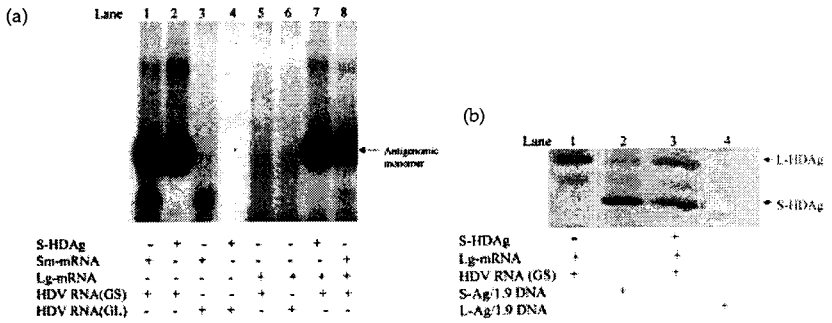

Fig. 3. Northern and Western blot analysis of HDV replication. (a) Detection of HDV antigenomic RNA from RNP transfection and mRNA cotransfection. The components of transfection are indicated as present (+) or absent (−). S-HDAg, recombinant S-HDAg purified from *E. coli*; Sm-mRNA, capped mRNA encoding S-HDAg; Lg-mRNA, capped mRNA encoding L-HDAg; HDV RNA (GS), 1·9 kb genomic RNA encoding the S-HDAg ORF; and HDV RNA (GL), 1 ·9 kb genomic RNA encoding the L-HDAg ORF. Antigenomic RNA was detected by Northern blotting at 5 days post-transfection. (b) Detection of the HDAg proteins in transfected COS-7 cells. Cell lysates were prepared at 5 days post-transfection and detected by anti-HDAg mAbs. Lanes: 1, cell lysate from cells transfected with L-HDAg-encoding capped mRNA together with genomic HDV RNA encoding S-HDAg; 2, cell lysate from cells transfected with the HDV cDNA plasmid containing the S-HDAg ORF; 3, transfection similar to that in lane 1 but with cotransfection of recombinant S-HDAg (derived from *E. coli*); and 4, cell lysate of cells transfected with the plasmid encoding L-HDAg.

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.

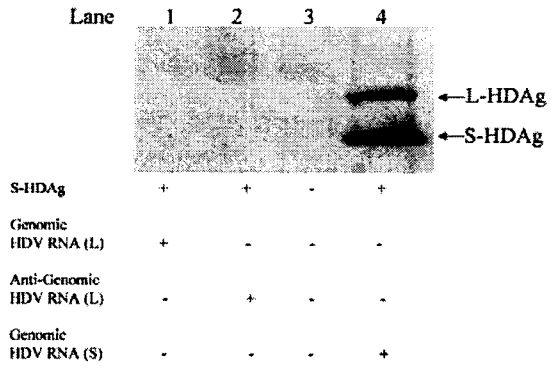

Fig. 4. Replication of L-HDAg-encoding RNA by RNP transfection, as detected by Western blot analysis of HDAg. The components of transfection are indicated as present (+) or absent (−). All RNAs are 1·9 kb in length. L, ORF for L-HDAg; S, ORF for S-HDAg; S-HDAg, recombinant S-HDAg purified from *E. coli*. Cell lysates were prepared at 5 days post-transfection.

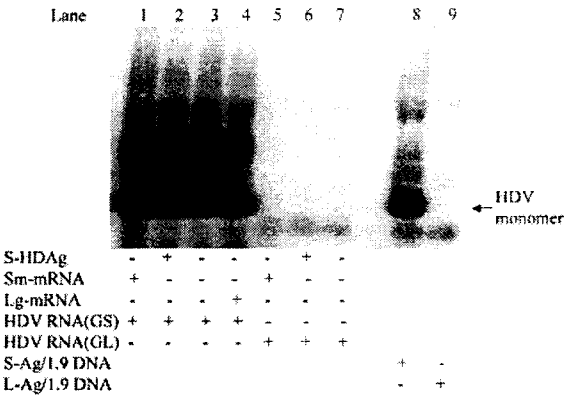

Fig. 5. Replication of HDV in TS δ3 cells, as detected by Northern blot analysis. *In vitro*-transcribed HDV genomic RNA containing either the S-HDAg or the L-HDAg ORF was transfected by either the RNA or RNP methods in cells stably expressing S-HDAg. Cellular RNA was harvested at 5 days post-transfection and the antigenomic HDV RNA was detected. The components of transfection are indicated.

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 early 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 TSδ3, 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.

I thank Dr M. M. C. Lai for his editorial help with the manuscript. This work was partially supported by the National Science Council, Taiwan, Republic of China; grant NSC-90-2320-B-040-029.

References

Bergmann, K. F. & Gerin, J. L. (1986). Antigens of hepatitis delta virus in the liver and serum of humans and animals. *Journal of Infectious Diseases* **154**, 702–706.

Bonino, F., Heermann, K. H., Rizzetto, M. & Gerlich, W. H. (1986). Hepatitis delta virus: protein composition of delta antigen and its hepatitis B virus-derived envelope. *Journal of Virology* **58**, 945–950.

Casey, J. L. & Gerin, J. L. (1995). Hepatitis D virus RNA editing: specific modification of adenosine in the antigenomic RNA. *Journal of Virology* **69**, 7593–7600.

Chang, F. L., Chen, P. J., Tu, S. J., Wang, C. J. & Chen, D. S. (1991). The large form of hepatitis delta antigen is crucial for assembly of hepatitis delta virus. *Proceedings of the National Academy of Sciences, USA* **88**, 8490–8494.

Chao, M. H. S. & Taylor, J. (1990). Role of two forms of hepatitis delta virus antigens: evidence for a mechanism of self-limiting genome replication. *Journal of Virology* **64**, 2357–2361.

Chou, H. C., Hsieh, T. Y., Sheu, G. T. & Lai, M. M. (1998). Hepatitis delta antigen mediates the nuclear import of hepatitis delta virus RNA. *Journal of Virology* **72**, 3684–3690.

Dingle, K., Bichko, V., Zuccola, H., Hogle, J. & Taylor, J. (1998). Initiation of hepatitis delta virus genome replication. *Journal of Virology* **72**, 4783–4788.

Glenn, J. S. & White, J. M. (1991). Trans-dominant inhibition of human hepatitis delta virus genome replication. *Journal of Virology* **65**, 2357–2361.

Gowans, E. J., Baroudy, B. M., Negro, F., Ponzetto, A., Purcell, R. H. & Gerin, J. L. (1988). Evidence for replication of hepatitis delta virus RNA in hepatocyte nuclei after *in vivo* infection. *Virology* **167**, 274–278.

Hwang, S. B. & Lai, M. M. (1993). A unique conformation at the carboxyl terminus of the small hepatitis delta antigen revealed by a specific monoclonal antibody. *Virology* **193**, 924–931.

Hwang, S. B., Lee, C. Z. & Lai, M. M. (1992). Hepatitis delta antigen expressed by recombinant baculoviruses: comparison of biochemical properties and post-translational modifications between the large and small forms. *Virology* **190**, 413–422.

Hwang, S. B., Jeng, K. S. & Lai, M. M. C. (1995). Studies of functional roles of hepatitis delta antigen in delta virus RNA replication. In *The Unique Hepatitis Delta Virus*, pp. 95–109. Edited by G. Dinter-Gottlieb. Berlin: Springer-Verlag.

Kuo, M. Y., Chao, M. & Taylor, J. (1989). Initiation of replication of the human hepatitis delta virus genome from cloned DNA: role of delta antigen. *Journal of Virology* **63**, 1945–1950.

Lai, M. M. (1995). The molecular biology of hepatitis delta virus. *Annual Review of Biochemistry* **64**, 259–286.

Lin, J. H., Chang, M. F., Baker, S. C., Govindarajan, S. & Lai, M. M. C. (1990). Characterization of hepatitis delta antigen: specific binding to hepatitis delta virus RNA. *Journal of Virology* **64**, 4051–4058.

Lo, K., Sheu, G. T. & Lai, M. M. (1998). Inhibition of cellular RNA polymerase II transcription by delta antigen of hepatitis delta virus. *Virology* **247**, 178–188.

Luo, G. X., Chao, M., Hsieh, S. Y., Sureau, C., Nishikura, K. & Taylor, J. (1990). A specific base transition occurs on replicating hepatitis delta virus RNA. *Journal of Virology* **64**, 1021–1027.

Modahl, L. E. & Lai, M. M. (1998). Transcription of hepatitis delta antigen mRNA continues throughout hepatitis delta virus (HDV) replication: a new model of HDV RNA transcription and replication. *Journal of Virology* **72**, 5449–5456.

Modahl, L. E. & Lai, M. M. (2000). The large delta antigen of hepatitis delta virus potently inhibits genomic but not antigenomic RNA synthesis: a mechanism enabling initiation of viral replication. *Journal of Virology* **74**, 7375–7380.

Modahl, L. E., Macnaughton, T. B., Zhu, N., Johnson, D. L. & Lai, M. M. (2000). RNA-dependent replication and transcription of hepatitis delta virus RNA involve distinct cellular RNA polymerases. *Molecular and Cellular Biology* **20**, 6030–6039.

Moraleda, G. & Taylor, J. (2001). Host RNA polymerase requirements for transcription of the human hepatitis delta virus genome. *Journal of Virology* **75**, 10161–10169.

Polson, A. G., Bass, B. L. & Casey, J. L. (1996). RNA editing of hepatitis delta virus antigenome by dsRNA-adenosine deaminase. *Nature* **380**, 454–456.

Rizzetto, M., Hoyer, B., Canese, M. G., Shih, J. W. K., Purcell, R. H. & Gerin, J. L. (1980). Delta agent: association of δ antigen with hepatitis B surface antigen and RNA in serum of δ-infected chimpanzees. *Proceedings of the National Academy of Sciences, USA* **77**, 6124–6128.

Ryu, W. S., Netter, H. J., Bayer, M. & Taylor, J. (1993). Ribonucleoprotein complexes of hepatitis delta virus. *Journal of Virology* **67**, 3281–3287.

Sheu, G. T. & Lai, M. M. (2000). Recombinant hepatitis delta antigen from *E. coli* promotes hepatitis delta virus RNA replication only from the genomic strand but not the antigenomic strand. *Virology* **278**, 578–586.

Wang, H. W., Wu, H. L., Chen, D. S. & Chen, P. J. (1997). Identification of the functional regions required for hepatitis D virus replication and transcription by linker-scanning mutagenesis of viral genome. *Virology* **239**, 119–131.

Xia, Y. P., Chang, M. F., Wei, D., Govindarajan, S. & Lai, M. M. (1990). Heterogeneity of hepatitis delta antigen. *Virology* **178**, 331–336.

Received 19 March 2002; Accepted 4 June 2002