JOURNAL OF VIROLOGY, Apr. 1993, p. 2228–2234
0022-538X/93/042228-07$02.00/0

Vol. 67, No. 4

Replication of Hepatitis Delta Virus RNA: Effect of Mutations of the Autocatalytic Cleavage Sites

THOMAS B. MACNAUGHTON,† YUEH-JU WANG,‡ AND MICHAEL M. C. LAI*

Howard Hughes Medical Institute and Department of Microbiology, University of Southern California School of Medicine, Los Angeles, California 90033-1054

Received 9 October 1992/Accepted 20 January 1993

Hepatitis delta virus (HDV) contains a circular RNA genome of 1.7 kb. HDV RNA replication is thought to proceed via a rolling-circle mechanism that is dependent on autocatalytic cleavage and ligation reactions. However, it has never been established that these ribozyme activities are indeed involved in HDV RNA replication. To investigate the possible biological significance of HDV RNA self-cleavage, we constructed several HDV dimer cDNAs containing single-base substitutions of the 3′ nucleotide of the genomic and the antigenomic self-cleavage sites. These mutations were known to affect self-cleavage in vitro to various extents. The effects of these mutations on HDV RNA replication were examined in hepatic and nonhepatic cell lines. The results showed that all of the mutants which had lost the in vitro self-cleavage activity could not replicate. The only mutant which retained full cleavage activity replicated as efficiently as the wild-type RNA. Thus, this study established that self-cleavage activity is required for HDV RNA replication in cells. Interestingly, the level of HDV RNA detected in cells transfected with this replication-competent mutant and that detected in cells transfected with the wild-type construct were similar in COS-7 cells but vastly different in HepG2 and Huh-7 cells, suggesting that HDV RNA self-cleavage activity may be modulated by cell-specific factors. We also compared the effects of mutations when the primary transcripts of these constructs were of either genomic or antigenomic sense. In constructs which synthesize primary transcripts of genomic sense, all of the antigenomic self-cleavage mutants produced as much hepatitis delta antigen (HDAg) as did the wild-type construct, even in the absence of detectable HDV RNA replication, whereas the genomic self-cleavage mutants produced very little HDAg. These and other data suggest that (i) the primary HDV RNA transcripts of both genomic and antigenomic polarities must first be processed to serve as a template for HDV RNA transcription, (ii) efficient cleavage at the antigenomic self-cleavage site is not required for HDAg expression, and (iii) HDV RNA replication most likely occurs by a double-rolling-circle mechanism.

Hepatitis delta virus (HDV) is a defective human pathogen that requires functions provided by either hepatitis B virus or the related hepadnavirus woodchuck hepatitis virus for its transmission (21, 22). The genome of HDV consists of a small, circular, single-stranded RNA of about 1.7 kb that is predicted to fold into an unbranched, rod-like structure because of a high potential for intramolecular base pairing (17, 26). While these features are unique for animal RNA viruses, they resemble those of plant pathogenic viroids and virusoids. Nevertheless, HDV RNA is much larger and, unlike viroid RNA, encodes a protein, the hepatitis delta antigen (HDAg). This protein consists of two related polypeptides, one of 24 kDa (small HDAg), which is essential for HDV RNA replication (12), and one of 27 kDa (large HDAg), which inhibits replication (6) but is essential for virus assembly (5).

Forms of HDV RNA extracted from infected livers (7) and transfected cell lines (12, 14) are consistent with a rolling-circle mechanism similar to that proposed for some viroids (2). This strategy is proposed to include RNA-directed RNA transcription from a circular genomic-sense RNA that leads to formation of a multimeric-length RNA replication intermediate, which is subsequently processed by autocatalytic cleavage into monomers. These monomer-size RNAs, which are complementary to the genome, then self-ligate to form circular RNAs, which will be referred to as the antigenome. Another round of rolling-circle replication from the antigenome generates progeny circular genomic RNA. However, many steps of this replication model have not been established, and it is conceivable that HDV RNA could go through only a single round of rolling-circle replication, with the multimeric, antigenomic RNA replication intermediate serving directly as the template for synthesis of genomic RNA.

In vitro studies have revealed that several regions on both the genomic and antigenomic strands of HDV RNA are capable of undergoing autocatalytic self-cleavage and self-ligation reactions (13, 23, 24, 29), a property also identified in a number of other naturally occurring RNA molecules, including virusoids and viroids. Presumably, these catalytic activities are involved in the cleavage of a multimeric-length RNA replication intermediate during the rolling-circle replication of HDV RNA. However, this possibility has never been established. The gene that encodes HDAg is located on the antigenomic strand of HDV RNA immediately upstream of the antigenomic cleavage site (10, 26), suggesting that cleavage at this site also plays a role in HDAg expression (10).

Work in our laboratory and others has defined the boundaries of the self-cleaving domains of HDV RNA, as well as suggesting the potential secondary and tertiary structures involved (3, 19, 20, 27, 28, 30). These studies have shown that several stem-loops and a possible pseudoknot structure are required for efficient cleavage of HDV RNA in vitro (3,

* Corresponding author.
† Present address: Division of Medical Virology, Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia 5000.
‡ Present address: Amgen Inc., Thousand Oaks, CA 91320.

20, 27, 30). However, these structures were determined from small subfragments of HDV RNA and appear to be inconsistent with the demonstrated rod structure for the entire HDV RNA (26). Indeed, in in vitro reactions, larger HDV RNA fragments cleaved considerably less efficiently than the minimum HDV RNA ribozymes (13, 19, 28, 29). Nevertheless, multimeric-length HDV RNA must cleave efficiently in vivo under physiological conditions if the self-cleavage reaction is involved in processing of an RNA replication intermediate. Why the constraints that restrict HDV RNA self-cleavage in vitro do not affect the process in vivo (i.e., during HDV RNA replication in HDV-infected cells) is unknown. The in vivo environment may be more favorable to formation of the RNA structure(s) conducive to self-cleavage; alternatively, the self-cleavage reaction is not involved in HDV RNA replication. Another possibility is that cellular factors modulate the self-cleavage in vivo. To examine these possibilities, we studied HDV RNA self-cleavage reactions in cells. However, in contrast to in vitro studies, it is difficult to separate the processes of self-cleavage and self-ligation to determine accurately the rate and extent of self-cleavage in vivo and it is difficult to rule out participation of cellular proteins in the cleavage reactions. Consequently, we made site-specific mutations on the nucleotides of the self-cleavage sites and examined their effects on HDV RNA replication in cells. This study showed that the mutations which affected the self-cleavage activity in vitro also inhibited the ability of the RNA to replicate. Thus, we conclude that self-cleavage activity is required for HDV RNA replication in vivo. This study also raises the possibility that cellular factors can modulate self-cleavage reactions and provides direct evidence that HDV RNA replicates predominantly by the double-rolling-circle mechanism.

MATERIALS AND METHODS

Site-directed mutagenesis. Site-directed mutagenesis was performed with a Muta-Gene kit (Bio-Rad) in accordance with the manufacturer's protocol, except that plasmid vector pBluescript SK± (Stratagene) containing a full-length cDNA of wild-type HDV RNA (17) inserted into the *Sal*I site was used to synthesize replicative-form DNA. The sequences of the mutagenesis primers were (i) for the genomic self-cleavage site (no. 384), 5′-CTTACCTGAT(C/A/T)GCCG GCATG-3′, and (ii) for the antigenomic self-cleavage site (no. 383), 5′-ATGCCGACC(T/G/A)GAAGAGGAAA-3′. These are complementary to nucleotides 679 to 698 and 894 to 913, respectively, of the antigenomic RNA of HDV (17). The final mutagenesis products were used to transform *Escherichia coli* DH-5α, and the resultant clones were screened by DNA sequencing.

Vectors and plasmid construction. All of the HDV cDNA used in this study encoded the small, 24-kDa, form of HDAg. Monomers of either wild-type HDV cDNA (17) or HDV cDNA carrying mutations in either the genomic or the antigenomic self-cleavage site, but not both, were inserted into the *Sal*I site of eukaryotic expression vector pECE1 (9) under control of the simian virus 40 early promoter. pECE-derived plasmids (see Fig. 1) were obtained by dimerization of HDV cDNA inserts by using a strategy involving partial *Sal*I digestion and religation with additional HDV cDNA monomers. All of these pECE-derived constructs were designed to synthesize genomic-sense and dimeric-length HDV RNA transcripts. In addition, the dimeric HDV cDNA inserts of several of the pECE-derived constructs (see Fig. 1) were removed by double digestion with restriction enzymes *Hin*dIII and *Xba*I, blunt ended, and recloned in both orientations into the *Sma*I site of transient expression vector pSVL (Pharmacia). These pSVL constructs allowed synthesis of HDV RNA transcripts of either polarity. For example, pSV-WT1 synthesizes genomic-sense transcripts and pSV-WT2 synthesizes antigenomic-sense transcripts. The construction of HDAg expression plasmid pTM5E, which contains an HDAg-coding open reading frame, has been described elsewhere (15). Plasmids pTMδSalA and pTMδSalB, which were used to generate HDV RNA strand-specific riboprobes (see below), contain a full-length copy of HDV cDNA (excised from pTMδNeo3A; 14) cloned in either orientation into the *Sal*I site of pGem3 (Promega).

Transfection. All transfections were mediated by Lipofectin (BRL) by a minor modification of the protocol recommended by the manufacturer. Briefly, 1 to 2 days prior to transfection, COS-7, Huh-7, and HepG2 cells were suspended in Dulbecco's minimum essential medium containing 5% fetal bovine serum and seeded into 24- or 6-well plates (Costar). When the cultures were 50 to 70% confluent, the medium was replaced with Dulbecco's minimum essential medium containing 1% fetal bovine serum, and 50 or 10 μl of a mixture containing 100 to 200 ng of plasmid DNA per μl and 500 ng of Lipofectin per μl was added per well to the 6- and 24-well plates, respectively. Following incubation overnight at 37°C in 5% CO_2, the medium was replaced with Dulbecco's minimum essential medium containing 5% fetal bovine serum and incubation was continued for an additional 3 or 5 days. Plasmid pTM5E, which contains the HDAg open reading frame, was usually included in the experiments to monitor efficiency of transfection by indirect immunofluorescence detection of HDAg. This was done with acetone-fixed cells grown on 13-mm-diameter glass coverslips and cultured in the 24-well plates at 4 days posttransfection, as described previously (31). In some experiments, pTM5E was included in the transfectants to provide HDAg in trans.

Northern (RNA) blot analysis. Total RNA was extracted from cells grown on 6-well plates 6 days after transfection by the guanidinium isothiocyanate procedure (8). The RNA was treated with formaldehyde, electrophoresed through formaldehyde-containing 1.1% agarose gels, blotted onto a nitrocellulose membrane (Hybond C extra; Amersham), and probed with ^{32}P-labeled HDV strand-specific riboprobes as described previously (14). Riboprobes for detecting genomic and antigenomic HDV RNA were transcribed, with T7 RNA polymerase, from pTMδSalB and pTMδSalA, respectively, following linearization of these plasmids at the *Pst*I site. Positive control HDV RNA was extracted from stable cell line N63, which was derived from COS-7 cells transfected with pTMδNeo3A (14).

Immunoblotting. Immunoblotting to detect HDAg was performed on cell lysates from 6-well plate cultures at 4 days after transfection. Polyacrylamide gel electrophoresis and protein transfer were performed as described previously (1). After transfer, the filters were incubated successively with a 1/1,000 dilution of high-titer rabbit anti-HDAg serum (31) followed by ^{125}I-labeled protein A (ICN). The small and large HDAg standards were provided by Yu-Ping Xia and consisted of extracts from stable cell lines S9 and L9, which express the small and large HDAgs, respectively.

RESULTS

Site-specific mutagenesis of the 3′ nucleotide of the self-cleavage sites. Previous studies with partial fragments of

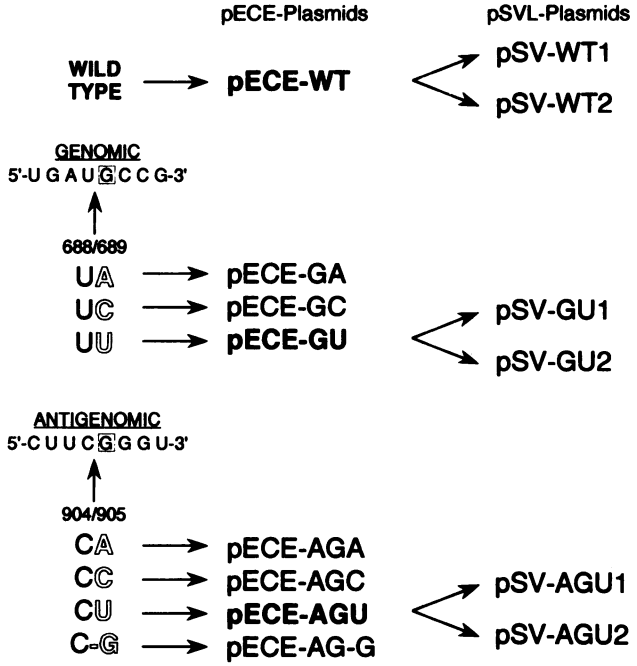

FIG. 1. HDV expression plasmids used in this study. All consisted of a dimer of wild-type HDV cDNA or HDV cDNA carrying mutations at the 3′ nucleotide of either the genomic or the antigenomic self-cleavage site. The pECE-derived plasmids were designed to synthesize primary transcripts of dimeric, genomic-sense HDV RNA. The pSVL-derived plasmids were designed to synthesize dimeric, genomic (1 suffix)- or antigenomic (2 suffix)-sense HDV RNA. The cleavage sites are indicated by arrows below the sequences (29).

HDV RNA have revealed that most mutations of the nucleotide at the 5′ side of the cleavage sites on both genomic and antigenomic HDV RNAs did not affect self-cleavage activity (19, 27), whereas most mutations of the 3′ G base almost completely eliminated the autocatalytic activity (27). To determine whether the self-cleavage activity of HDV RNA is involved in HDV RNA replication, we performed site-specific mutagenesis of the self-cleavage site to correlate self-cleavage activity with the ability of HDV RNA to replicate. We targeted the 3′ G base of both the genomic and the antigenomic self-cleavage sites for mutagenesis. In all, seven mutants were obtained, three at the genomic self-cleavage site, in which the 3′ G was converted to A, C, or U (giving rise to plasmids pECE-GA, pECE-GC, and pECE-GU, respectively; Fig. 1), and four at the antigenomic self-cleavage site, in which the 3′ G was converted to A, C, or U or deleted completely (giving rise to plasmids pECE-AGA, pECE-AGC, pECE-AGU, and pECE-AG-G, respectively; Fig. 1). These site-specific mutations were constructed into dimeric copies of HDV cDNA and placed under an simian virus 40 T-antigen promoter such that genomic-sense HDV RNA of dimeric length would be transcribed from the plasmids.

Northern blot analysis of self-cleavage mutants. Dimers of HDV cDNA with mutations in either the genomic or the antigenomic self-cleavage sites were transfected into COS-7 cells. To guard against the possibility that mutations of the self-cleavage sites would interfere with HDAg mRNA processing and thus inhibit expression of HDAg, some initial experiments were performed in which these plasmids were cotransfected with HDAg-p24 expression plasmid pTM5E. However, since all of the mutants permitted HDAg expression (see below), pTM5E was omitted from later experiments, except where required to measure transfection efficiency. Total cellular RNA from the transfected cells was examined for HDV RNA by Northern blot hybridization. Detection of genomic-sense HDV RNA from COS-7 cells transfected with the different plasmids is shown in Fig. 2. Comparable results were obtained for antigenomic HDV RNA (data not shown). Monomeric HDV RNA was detected in cells transfected with the wild-type construct (pECE-WT). A small amount of dimeric HDV RNA was also seen. Among all of the cleavage site mutants, however, only the genomic self-cleavage site mutant, which had a 3′ G-to-A mutation (pECE-GA), synthesized detectable amounts of HDV RNA. The levels of RNA replication were similar in the wild type and this mutant. In contrast, none of the other

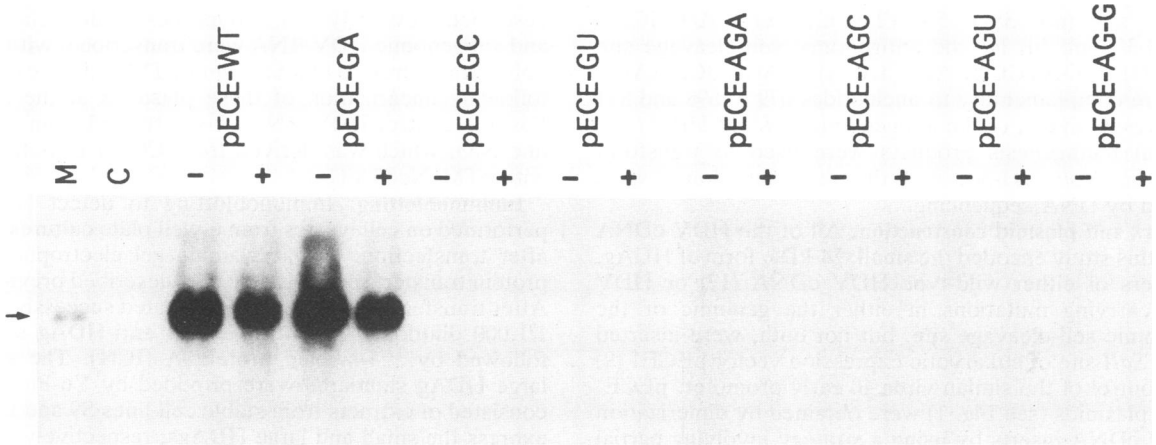

FIG. 2. Northern blot analysis of HDV RNA in COS-7 cells transfected with pECE-derived expression plasmids. Cells were transfected with wild-type HDV cDNA or various mutant HDV cDNAs with (+) or without (−) cotransfected HDAg expression plasmid pTM5E. RNA was detected by an antigenomic HDV RNA probe. Lane C contained mock-transfected COS-7 cells, and lane M contained total RNA extracted from stable, HDV RNA-positive cell line N63. The arrow at the left indicates monomeric-length HDV RNA. All of the lanes, except M (2 μg), contained 15 μg of total cellular RNA. Autoradiographic exposure time was 16 h.

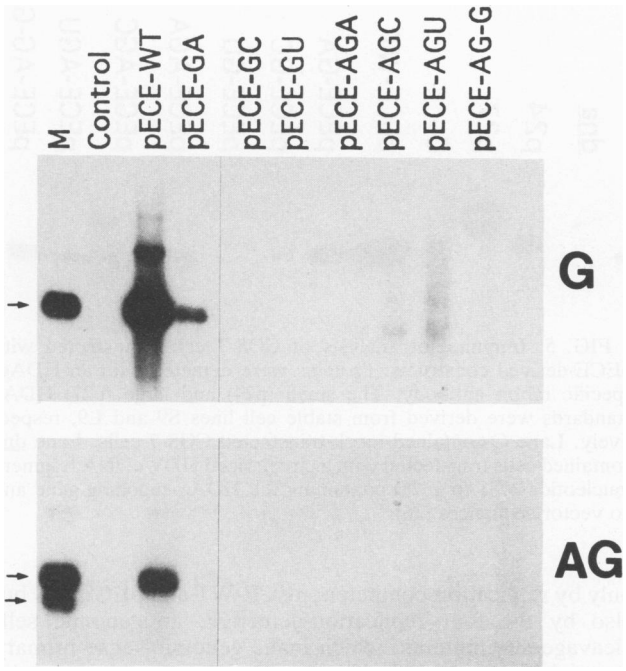

FIG. 3. Northern blot analysis of Huh-7 cells transfected with pECE-derived expression plasmids. Cells were transfected with wild-type or mutant HDV cDNA without cotransfected pTM5E. Either genomic (G) or antigenomic (AG) HDV RNA was detected. The control lane contained mock-transfected Huh-7 cells, and lane M contained a total RNA extract from N63 cells. All of the lanes contained 15 μg of total cellular RNA, and the autoradiographic exposure time was 16 h. The arrows at the left indicate monomeric-length HDV RNA (both panels) and HDAg mRNA (AG panel only).

cleavage site mutants synthesized detectable levels of RNA, indicating that these RNAs could not replicate. Identical results were obtained with or without cotransfection of plasmid pTM5E (Fig. 2); thus, the failure of their replication was not due to the possible effects of mutations on the synthesis or properties of HDAg. Significantly, among all of the genomic and antigenomic cleavage site mutants, only the genomic 3′ G-to-A mutant retained a significant level of self-cleavage activity in vitro (26a, 27). These results suggested strongly that the self-cleavage activity of HDV RNAs of both genomic and antigenomic senses is required for HDV RNA replication.

Cell type-specific differences in the replication of cleavage-site mutants. To determine whether the cleavage efficiency of HDV RNA varies with cell type, we transfected two hepatoma-derived cell lines, Huh-7 and HepG2, with the wild type or the various cleavage site mutant plasmids. Patterns of HDV RNA replication similar to that in COS-7 cells were observed in Huh-7 and HepG2 cells; i.e., only the wild type and the pECE-GA mutant replicated. However, while COS-7 cells transfected with pECE-GA showed a level of HDV RNA similar to that detected in cells transfected with pECE-WT, the level of HDV RNA replication in Huh-7 cells by pECE-GA was consistently much lower (less than 1/10) than that of pECE-WT in the same cells (Fig. 3). This difference was observed in both genomic and antigenomic sense RNAs. Similar results were also obtained with HepG2 cells (data not shown). This result suggests that cell-specific factors modulate HDV RNA self-cleavage in cells. Preliminary quantitative analysis suggested that these cell-specific differences were due to enhancement of wild-type HDV RNA replication in the hepatoma-derived cell lines (Huh-7 and HepG2 cells) over that in COS-7 cells, rather than inhibition of replication of pECE-GA mutant RNA in the hepatoma cell lines. Specifically, roughly equivalent levels of wild-type HDV RNA replication were detected in Huh-7 (and HepG2) cells and in COS-7 cells (compare pECE-WT-transfected lanes in Fig. 2 and 3), although (i) the expression plasmid (pECE) contains a simian virus 40 origin of replication, leading to amplification of plasmids in COS-7 cells, and (ii) higher transfection efficiencies were obtained in COS-7 cells (approximately 30 to 50%) than in the hepatoma cell lines (approximately 10 to 15%).

Faint bands of genomic-sense monomer HDV RNA were detected in Huh-7 cells transfected with some of the antigenomic self-cleavage mutants (Fig. 3, top panel). These bands probably represent the processed RNA of the primary genomic-sense transcripts from the transfected plasmids, since the genomic-sense transcripts likely retained the cleavage activity. In contrast, monomer RNA was not detected with the two genomic cleavage-defective mutants (pECE-GC and pECE-GU; Fig. 3, top panel), consistent with the defectiveness of their cleavage activity. Interestingly, with these two genomic cleavage site mutants, no antigenomic-sense monomer HDV RNA was detected either (Fig. 3, bottom panel), indicating that the unprocessed dimeric genomic-sense transcripts from these plasmids were not used as templates for transcription of antigenomic-sense HDV RNA, which would have been cleaved at a normal rate.

RNA replication of cleavage site mutants in different polarities. The results shown above indicated that self-cleavage is involved in HDV RNA replication, which likely occurs by a rolling-circle mechanism. To distinguish further between a double-rolling-circle mechanism, in which both genomic and antigenomic RNAs are transcribed from the circular RNA template, and a single-rolling-circle mechanism of replication, in which one of the multimeric RNA replication intermediates directly serves as a template for RNA replication, we constructed several pairs of cleavage site mutants, each of which expresses the same RNA in the opposite senses. Recombinant plasmids pSV-WT1, pSV-GU1, and pSV-AGU1 were designed to synthesize primary transcripts of genomic polarity, while pSV-WT2, pSV-GU2, and pSV-AGU2 generate the corresponding RNAs of antigenomic polarity (Fig. 1). If HDV RNA replicates by a single-rolling-circle mechanism, e.g., the genomic RNA is directly transcribed from the multimeric antigenomic RNA template, pSV-GU2 should be able to replicate, since the primary transcript (antigenomic sense), which cannot be processed, should be transcribed into genomic RNA and processed normally; but pSV-GU1 is not expected to replicate. On the contrary, if the antigenomic RNA is transcribed from the multimeric genomic RNA, pSV-AGU1 should replicate, but pSV-AGU2 will not. These constructs were transfected into COS-7 cells, and total RNA was examined by Northern blot hybridization (Fig. 4).

The results showed that the genomic and antigenomic transcripts of the wild-type constructs (pSV-WT1 and pSV-WT2) led to similar levels of HDV RNA replication, indicating that primary transcripts of both polarities could initiate HDV RNA replication with equal efficiencies (Fig. 4). In contrast, neither of the genomic or antigenomic transcripts of the cleavage site mutants led to an appreciable level of RNA replication. These results indicated that self-cleavage of both genomic and antigenomic strands is required for

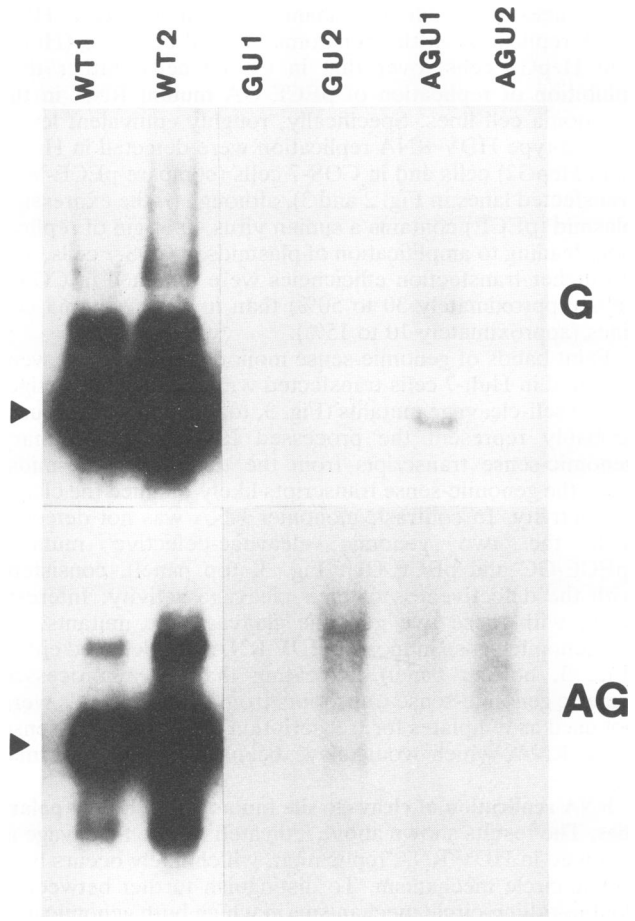

FIG. 4. Northern blot analysis of HDV RNA in COS-7 cells transfected with pSVL-derived expression plasmids. Cells were transfected with the wild-type or various mutant pSVL plasmids (Fig. 1). The polarity of the primary transcripts from these plasmids was genomic (WT1, GU1, and AGU1) or antigenomic (WT2, GU2, and AGU2). Either genomic (G)- or antigenomic (AG)-sense HDV RNA was detected. The arrowheads show the positions of monomeric-length HDV RNA. All of the lanes contained 15 μg of total cellular RNA. Autoradiographic exposure time was 16 h.

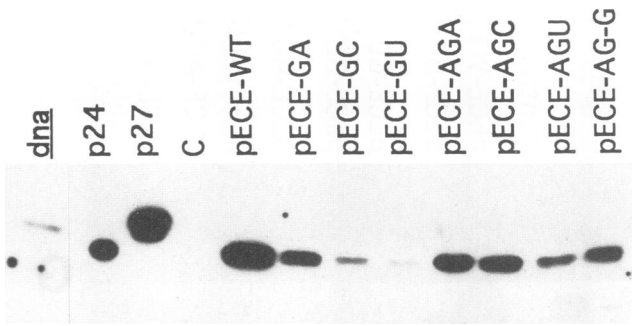

FIG. 5. Immunoblot analysis of COS-7 cells transfected with pECE-derived constructs. Proteins were detected with an HDAg-specific rabbit antibody. The small (p24) and large (p27) HDAg standards were derived from stable cell lines S9 and L9, respectively. Lane C contained mock-transfected COS-7 cells. Lane dna contained cells transfected with a circularized HDV cDNA fragment (nucleotides 781 to 1678) containing the HDAg-encoding gene and no vector sequences (16).

HDV RNA replication. Therefore, HDV RNA replication most likely occurs by a double-rolling-circle mechanism. Nevertheless, a small amount of genomic-sense monomer RNA from pSV-AGU1 was detected and a small amount of antigenomic-sense monomer RNA from pSV-GU2 was detected. These RNAs likely represent the cleavage products of the respective primary transcripts, since these RNAs should have normal cleavage activity. In contrast, no corresponding RNA from pSV-AGU2 or pSV-GU1 was detected. These data further support the notion that the unprocessed dimeric HDV RNAs of either sense from the primary transcripts were unable to serve as templates for HDV RNA replication. These results are consistent with the double-rolling-circle model of HDV RNA replication.

Expression of HDAg in transfected cells. To confirm the double-rolling-circle model of HDV RNA replication, COS-7 cells transfected with either pECE-WT or the various self-cleavage site mutants were examined by immunoblotting to detect HDAg synthesis (Fig. 5). Surprisingly, similar, although not identical, levels of HDAg were synthesized not only by replication-competent pECE-WT and pECE-GA but also by the four replication-defective, antigenomic self-cleavage site mutants, which make genomic-sense primary transcripts. Since HDAg is translated from antigenomic-sense RNA, this result further suggested that the primary genomic-sense HDV RNA transcripts from these mutants were processed correctly and were transcribed into antigenomic-sense HDV RNA. In contrast, the two replication-defective genomic self-cleavage site mutants (pECE-GC and pECE-GU) synthesized only a small amount of HDAg (less than 5%). Since these two mutants made genomic-sense primary transcripts which could not be cleaved, these results further suggested that the unprocessed RNA could not serve as a template for synthesis of antigenomic-sense HDV RNA or HDAg mRNA. The small amount of HDAg detected most likely was made by HDV RNA transcribed from an endogenous promoter of HDV cDNA (16), since an HDV cDNA fragment containing the complete HDAg open reading frame also expressed a similar amount of HDAg (Fig. 5, labeled dna). These results further suggest that only the processed monomer (probably circularized) HDV RNA can be replicated, thus supporting the double-rolling-circle mechanism.

DISCUSSION

This study was the first to examine, although indirectly, HDV RNA self-cleavage in vivo. This was done by determining the effects of mutations of the 3′ nucleotide of the genomic and antigenomic self-cleavage sites on HDV RNA replication and HDAg expression. It was found that loss of in vitro self-cleavage activity correlated with inhibition of HDV RNA replication in vivo. Thus, self-cleavage of HDV RNA is required for replication of HDV RNA. This is the first evidence that RNA self-cleavage is involved in RNA replication. Although we did not directly demonstrate the self-cleavage in vivo, the correlation of in vitro self-cleavage activity with RNA replication in seven different mutants makes it highly unlikely that these mutations affect other, unknown RNA functions.

Our studies also demonstrated that HDV RNA replicates via a double-rolling-circle mechanism. Previously, detection of multimeric forms of HDV RNA of both polarities in HDV-infected livers (7) and in transfected cell cultures (12, 14), along with the demonstration in vitro of autocatalytic

self-cleavage and self-ligation activities of HDV RNAs of both polarities (13, 23, 24, 29), suggested a rolling-circle mechanism. It was unclear, however, whether HDV RNA replication could proceed via a multimeric linear replicative intermediate RNA, i.e., a single-rolling-circle mechanism, such as is thought to occur for viroids and virusoids that have only one self-cleavage site (25). Our study shows that replication of both genomic and antigenomic HDV RNAs requires a processed monomer RNA template and thus provides direct evidence that HDV replicates predominantly by a double-rolling-circle mechanism. However, we cannot rigorously exclude the possibility that the cleavage site mutants underwent a low level of HDV RNA replication, as suggested by the low level of HDAg expression by the replication-defective genomic self-cleavage site mutants (although the HDAg mRNA of these mutants may have been transcribed directly from the endogenous promoter of HDV cDNA; 16). Interestingly, the antigenomic self-cleavage mutants and the wild-type construct produced comparable levels of HDAg, even in the absence of HDV RNA replication. This finding confirms and extends a previous suggestion indicating that self-cleavage at the antigenomic site is not required for HDAg expression (11). It should be pointed out that the primary dimeric transcripts from various mutants were, in general, not detected in the cells when no RNA cleavage or replication occurred. It is likely that the unprocessed transcripts were unstable. Thus, most of the HDV RNA species detected in transfected cells most likely represents the replicated RNA.

Mutational analysis and RNase digestion studies of HDV ribozymes have revealed that the structure, rather than the sequence, of these domains is important for self-cleavage; furthermore, they suggest that the active conformers are not the most stable form predicted by computer analysis (20, 28, 30). The latter finding is consistent with the enhancement of cleavage by repeated cycles of denaturation and renaturation, and by incubation at high temperatures or in the presence of denaturants, since these conditions may alter the structure of HDV RNA (28) or increase the rate of interconversion of the various RNA conformers (19). In HDV-infected cells, the most stable RNA conformers must be converted to the catalytically active form by changing the physiological environment or by cellular factors. These cellular factors may fulfill the role(s) played by the denaturants in in vitro self-cleavage reactions. Our current study indeed provides evidence suggesting that HDV RNA self-cleavage in vivo can be modulated by cell-specific factors. Consistent with this notion, a protein-mediated increase in the efficiency of in vivo self-splicing of some group 1 introns has been noted (4) and a recent preliminary study has shown that nuclear extracts accelerated the rate of HDV RNA *trans*-cleavage in vitro (3). Furthermore, since the RNA structures required for self-cleavage of HDV ribozymes in vitro are different from the demonstrated rod structure for the entire HDV RNA, it is imperative that some mechanisms exist to facilitate the transition from the rod to the active ribozyme structure. Since a number of different cell types of diverse animal origins have been shown to support HDV RNA replication following HDV cDNA transfection, the cellular factors that affect self-cleavage may be ubiquitous in nature. However, since the highest level of HDV RNA replication was detected in human hepatocyte-derived cell lines, these factors may be optimal in hepatocytes. This possibility may explain why HDV infection was detected only in woodchuck hepatitis virus-infected woodchuck livers and not in extrahepatic sites infected with woodchuck hepatitis virus (18). Future studies should attempt to identify the cell factors modulating self-cleavage, as this may ultimately lead to the design of novel antiviral agents to combat this highly pathogenic agent.

ACKNOWLEDGMENTS

We thank E. J. Gowans for comments on the manuscript and Y.-P. Xia for supplying cell lines S9 and L9. We also thank H.-N. Wu of Academia Sinica, Taiwan, for sharing unpublished data on in vitro cleavage site mutants.

This work was supported by grants from the Public Health Service (AI26741) and the National Health and Medical Research Council of Australia. T.B.M. was a Research Associate and M.M.C.L. is an Investigator of the Howard Hughes Medical Institute.

REFERENCES

1. **Bergmann, K. F., and J. L. Gerin.** 1986. Antigens of hepatitis delta virus in the liver and serum of humans and animals. J. Infect. Dis. **154**:702–706.
2. **Branch, A. D., and H. D. Robertson.** 1984. A replication cycle for viroids and other small infectious RNA's. Science **223**:450–455.
3. **Branch, A. D., and H. D. Robertson.** 1991. Efficient trans cleavage and a common structural motif for the ribozymes of the human hepatitis δ agent. Proc. Natl. Acad. Sci. USA **88**:10163–10167.
4. **Cech, T. R.** 1990. Self-splicing of group I introns. Annu. Rev. Biochem. **59**:543–568.
5. **Chang, F.-L., P.-J. Chen, S.-J. Tu, C.-J. Wang, and D.-S. Chen.** 1991. The large form of hepatitis delta antigen is crucial for the assembly of hepatitis delta virus. Proc. Natl. Acad. Sci. USA **88**:8490–8494.
6. **Chao, M., S.-Y. Hsieh, and J. Taylor.** 1990. Role of two forms of hepatitis delta virus antigen: evidence for a mechanism of self-limiting genome replication. J. Virol. **64**:5066–5069.
7. **Chen, P.-J., G. Kalpana, J. Goldberg, W. Mason, B. Werner, J. L. Gerin, and J. Taylor.** 1986. Structure and replication of the genome of the hepatitis delta virus. Proc. Natl. Acad. Sci. USA **83**:8774–8778.
8. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
9. **Ellis, L., E. Clauser, D. O. Morgan, M. Edery, R. A. Roth, and W. J. Rutter.** 1986. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell **45**:721–732.
10. **Hsieh, S.-Y., M. Chao, L. Coates, and J. Taylor.** 1990. Hepatitis delta virus genome replication: a polyadenylated mRNA for delta antigen. J. Virol. **64**:3192–3198.
11. **Hsieh, S.-Y., and J. Taylor.** 1991. Regulation of polyadenylation of hepatitis delta virus antigenomic RNA. J. Virol. **65**:6438–6446.
12. **Kuo, M. Y.-P., M. Chao, and J. Taylor.** 1989. Initiation of replication of the human hepatitis delta virus genome from cloned DNA: role of delta antigen. J. Virol. **63**:1945–1950.
13. **Kuo, M. Y.-P., L. Sharmeen, G. Dinter-Gottlieb, and J. Taylor.** 1988. Characterization of self-cleaving RNA sequences on the genome and antigenome of human hepatitis delta virus. J. Virol. **62**:4439–4444.
14. **Macnaughton, T. B., E. J. Gowans, A. R. Jilbert, and C. J. Burrell.** 1990. Hepatitis delta virus RNA, protein synthesis and associated cytotoxicity in a stably transfected cell line. Virology **177**:692–698.
15. **Macnaughton, T. B., E. J. Gowans, B. Reinboth, A. R. Jilbert, and C. J. Burrell.** 1990. Stable expression of hepatitis delta virus antigen in a eukaryotic cell line. J. Gen. Virol. **71**:1339–1345.
16. **Macnaughton, T. B., and M. M. C. Lai.** Identification of promoters of HDV RNA transcription on HDV cDNA. *In* Proceedings of the Fourth International Symposium on Hepatitis Delta Virus and Liver Disease, in press.
17. **Makino, S., M.-F. Chang, C. K. Shieh, T. Kamahora, D. M.**

Vannier, S. Govindarajan, and M. M. C. Lai. 1987. Molecular cloning and sequencing of a human delta virus RNA. Nature (London) **329:**343–346.

18. **Negro, F., B. E. Korba, B. Forzani, B. M. Baroudy, T. L. Brown, J. L. Gerin, and A. Ponzetto.** 1989. Hepatitis delta virus (HDV) and woodchuck hepatitis virus (WHV) nucleic acids in tissues of HDV-infected chronic WHV carrier woodchucks. J. Virol. **63:**1612–1618.
19. **Perrotta, A. T., and M. D. Been.** 1990. The self-cleaving domain from the genomic RNA of hepatitis delta virus: sequence requirements and the effects of denaturant. Nucleic Acids Res. **18:**6821–6827.
20. **Perrotta, A. T., and M. D. Been.** 1991. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. Nature (London) **350:**434–436.
21. **Ponzetto, A., P. J. Cote, H. Popper, B. H. Hoyer, W. T. London, E. C. Ford, F. Bonino, R. H. Purcell, and J. L. Gerin.** 1984. Transmission of the hepatitis B virus-associated delta agent to the eastern woodchuck. Proc. Natl. Acad. Sci. USA **81:**2208–2212.
22. **Rizzetto, M.** 1983. The delta agent. Hepatology **3:**729–737.
23. **Sharmeen, L., M. Y.-P. Kuo, G. Dinter-Gottlieb, and J. Taylor.** 1988. Antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. J. Virol. **62:**2674–2679.
24. **Sharmeen, L., M. Y.-P. Kuo, and J. Taylor.** 1989. Self-ligating RNA sequences on the antigenome of human hepatitis delta virus. J. Virol. **63:**1428–1430.
25. **Symons, R. H.** 1989. Self-cleavage of RNA in the replication of small pathogens of plants and animals. Trends Biochem. Sci. **14:**445–450.
26. **Wang, K.-S., Q.-L. Choo, A. J. Weiner, J.-H. Ou, R. C. Najarian, R. M. Thayer, G. T. Mullenbach, K. J. Denniston, J. L. Gerin, and M. Houghton.** 1986. Structure, sequence and expression of the hepatitis (δ) viral genome. Nature (London) **323:**508–514. [Author's correction, Nature (London) **328:**456, 1987.]

26a. **Wu, H.-N.** Personal communication.

27. **Wu, H.-N., and Z.-S. Huang.** 1992. Mutagenesis analysis of the self-cleavage domain of hepatitis delta virus antigenomic RNA. Nucleic Acids Res. **20:**5937–5941.
28. **Wu, H.-N., and M. M. C. Lai.** 1990. RNA conformational requirements of self-cleavage of hepatitis delta virus RNA. Mol. Cell. Biol. **10:**5575–5579.
29. **Wu, H.-N., Y.-J. Lin, F.-P. Lin, S. Makino, M.-F. Chang, and M. M. C. Lai.** 1989. Human hepatitis δ virus RNA subfragments contain an autocleavage activity. Proc. Natl. Acad. Sci. USA **86:**1831–1835.
30. **Wu, H.-N., Y.-J. Wang, C.-F. Hung, H.-J. Lee, and M. M. C. Lai.** 1992. Sequence and structure of the catalytic RNA of hepatitis delta virus genomic RNA. J. Mol. Biol. **223:**233–245.
31. **Xia, Y.-P., C.-T. Yeh, J.-H. Ou, and M. M. C. Lai.** 1992. Characterization of nuclear targeting signal of hepatitis delta antigen: nuclear transport as a protein complex. J. Virol. **66:**914–921.