Identification and Characterization of a Hepatitis Delta Virus RNA Transcriptional Promoter

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Transcription and replication of hepatitis delta virus (HDV) RNA are performed by the cellular enzyme RNA polymerase II (Pol II). As DNA is the normal template for Pol II, the enzyme must undergo template switching. The mechanism for this is unknown, but since HDV RNA can form a rod-like molecule by extensive intramolecular base pairing, it has been suggested that a double-stranded region(s) of HDV RNA serves as a recognition site for Pol II. A bidirectional promoter has been identified previously on HDV cDNA (T. B. Macnaughton, M. R. Beard, M. Chao, E. J. Gowans, and M. M. C. Lai, Virology 196:629–636, 1993). In the present study, genomic RNA corresponding to this region was able to direct the synthesis of antigenomic RNA in a nuclear extract transcription reaction, whereas genomic RNA species containing a mutation that resulted in a replication-defective phenotype were unable to do so. Thus, this region, the location of which is defined as nucleotides 1608 to 1669 on the basis of a highly conserved structure, represents a RNA-RNA promoter. Computer analysis of the RNA secondary structure predicted that the promoter contains two bulge regions in a stem-loop structure which encompasses a GC-rich motif. This predicted model was confirmed by enzyme digestion and primer extension analysis. The promoter is located at one end of the rod and has some homology with the simian virus 40 late promoter. A number of other mutations were introduced within this region, and expression plasmids were constructed to examine the effects of mutations in the promoter on HDV RNA replication. Disruption of the overall secondary structure, particularly the bulge regions, totally inhibited HDV RNA replication.

Hepatitis delta virus (HDV) is a defective virus that is dependent on concurrent infection with hepatitis B virus for replication and expression. The HDV genome comprises a single-stranded circular RNA molecule of 1.7 kb that has the potential to fold into a stable unbranched rod-like structure as a result of a high degree of intramolecular base pairing (24, 41). Replication of HDV RNA occurs in the nuclei of infected cells, most likely by a double rolling-circle mechanism in which there is no DNA intermediate. HDV RNA has the ability for in vitro self-cleavage and ligation at specific sites, and as mutation of these sites results in loss of virus replication, it is likely that these sites are vital for genome replication (34). HDV replication is also dependent on expression of the small form of hepatitis delta antigen (S-HDAg; p24), although the function of the antigen in the replication process is still unclear (25). In contrast, the large form of HDAg (L-HDAg; p27) inhibits HDV replication but is essential for packaging and export (8). An 800-nucleotide (nt) poly(A)"""" RNA species that represents the mRNA for HDAG has been isolated from HDV-infected liver tissue (11, 18) and identified in cDNA-transfected cell lines (23, 31). The 5' end of this transcript, which is complementary to the virion RNA (i.e., antigenic), was mapped to position 1631, 33 nt upstream of the HDAG translation initiation codon (18). Genomes which encode only L-HDAg are noninfectious, and L-HDAg is expressed as the result of a specific RNA editing event which was originally thought to occur on the genomic HDV RNA (5, 42) but is now considered to occur on the antigenomic RNA (7). This editing event leads to the conversion of an amber stop codon to a tryptophan codon at codon position 195 in the HDAg mRNA and results in a 19-amino-acid extension at the carboxy terminus of the S-HDAg to produce L-HDAg. Although the editing event may not necessarily require HDV RNA replication, the appearance of L-HDAg is often used as a marker of HDV RNA replication (29).

Initial transfection experiments to study HDV replication used trimeric or dimeric forms of HDV cDNA under the control of a foreign promoter. More recently, linearized, monomeric HDV cDNA as well as recircularized HDV cDNA have been shown to initiate HDV RNA replication (30, 39), and HDV cDNA was shown to contain an endogenous promoter which can direct HDV RNA transcription. This promoter was found to be bidirectional and is located in a position that corresponds to the end of the HDV rod structure, upstream of the HDAg coding region (27).

A previous study from our laboratory suggested, on the basis of α-amanitin sensitivity, that HDV RNA transcription and replication were performed by RNA polymerase II (Pol II) (32), and this has since been confirmed (14). Consequently, Pol II must undergo template switching from DNA to RNA, although the mechanism for this is unknown. The ability of HDV RNA to fold into a stable rod structure with regions of double-stranded RNA may facilitate this switch.

The aim of this study was to determine if the previously described promoter region located on HDV cDNA has a similar function on HDV RNA. RNA from this region or from a control region was added to an in vitro transcription reaction, and the nature of the products was examined. Mutations were induced in this region by PCR mutagenesis, and the effects on in vitro transcription were correlated with HDV RNA replica-
tion. We have also correlated the effects of these mutations on RNA secondary structure with HDV replication.

MATERIALS AND METHODS

HDV expression plasmids. Single-point and multiple mutations in the putative promoter region were introduced by PCR mutagenesis. Mutant primers M1 to M9 (Table 1) were synthesized so as to contain the desired mutation and a HindIII site at the 5′ end to facilitate cloning. These mutant primers were used in a PCR with a common primer which contained a 5′ KpnI site to permit identification. The PCR was performed in a 100-μl volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 50 μM each deoxynucleoside triphosphate, 3 mM MgCl₂, 1.25 U of Taq DNA polymerase (Perkin-Elmer Cetus), and 1 ng of HDV cDNA template (pTM27; full-length HDV cDNA in pGEM-3 [Promega] [33]). The reaction mix was heated to 94°C for 1 min, followed by 27 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by 27 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and finally 72°C for 7 min, were performed. The PCR products were analyzed by gel electrophoresis and purified by using Geneclean (Bio 101). The purified products were digested with HindIII and KpnI and directionally cloned into the vector pECE-8/C (Fig. 1A) to generate mutant constructs pECEM1 to 9. This resulted in substitution of the wild-type HDV sequence with a sequence containing the mutation in the putative promoter region. All constructs were sequenced by substitution of the wild-type HDV sequence with a sequence containing the mutation in the putative promoter region. All constructs were sequenced by using an Applied Biosystems automated sequencer.

The HDV expression plasmid pECE-8/C (Fig. 1A) was constructed by blunt-end cloning 1.3 copies of HDV cDNA, removed by BamHI-SacI double digestion from the plasmid pCl (33), and then ligated into the HindIII and EcoRI sites of the eukaryotic transient expression plasmid pECE (12).

Plasmid pBHVD199, which contains 199 nt of wild-type HDV cDNA (nt 1540 to 60), and plasmids pBHVM1 to M9, containing the mutations indicated in Table 1, were generated in a PCR in which primer PE1 (5′CGCAGGCTTACGTCGAGGATCCTCTCGGGAGC; bases in italics represent a SacI site) and PE2 (5′GCCTCACATCTCCCTCTCCGGGAGC; bases in italics represent a KpnI site) were annealed to the HDV cDNA construct pECE-8/C or pECE-8/C/M1 to M9, respectively. The restriction sites were incorporated into the 5′ end of the primers to facilitate directional cloning into pBlueScript KS+ (Strategene). These plasmids direct the transcription of a 199-nt genomic HDV RNA (RNA199; Fig. 2) from the T7 promoter site and were engineered to transcribe HDV RNA with minimum vector sequences.

Plasmid pMB37 (16) contains the 5′ flanking fragment of HDV cDNA (nt 481 to 1109) and directs the transcription of a 628-nt genomic HDV RNA (RNA600; Fig. 2) molecule transcribed from the T7 promoter site.

Transfection. All transfections were performed with DOTAP (Boehringer Mannheim) by a previously described modification of the manufacturer’s protocol (30). Briefly, the growth medium in 50 to 70% confluent Cos7 and HuH7 cells seeded in six-well plates and on coverslip cultures was replaced with Dulbecco’s minimum essential medium containing 1% fetal bovine serum and transfected with 5 and 1 μg of DNA, respectively. Following incubation overnight at 37°C in 5% CO₂, the medium was replaced with Dulbecco’s minimal essential medium containing 5% fetal bovine serum and incubated for a further 4 to 6 days. The transfection efficiency was assessed by detection of HDAg by a direct immunofluorescence assay (31).

Northern (RNA) blot hybridization. Five to six days posttransfection, total RNA was extracted by using a guanidinium thiocyanate-phenol-chloroform protocol, separated electrophoretically in formaldehyde-containing agarose gels, transferred onto a nitrocellulose membrane (Hybond C extra; Amersham), and probed with 32P-labeled HDV strand-specific RNA probes as described previously (32). RNA extracted from H189 cells (33) was used as an HDV positive control.

Quantification of the specific 1.7-kb HDV RNA species from Northern blot hybridization was carried out with the Molecular Dynamics densitometer com-

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**TABLE 1. HDV-specific oligonucleotides**

<table>
<thead>
<tr>
<th>Position</th>
<th>Designation</th>
<th>Sequence&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1650–1679</td>
<td>Wild type</td>
<td>TTTAAGCTTGTGCGTCGGGCG CGGAGTCCAG CAGTCT</td>
</tr>
<tr>
<td>M1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M2</td>
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<tr>
<td>1620–1679</td>
<td></td>
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</tr>
<tr>
<td>1650–1679</td>
<td></td>
<td></td>
</tr>
<tr>
<td>479–499</td>
<td>Common primer</td>
<td>CCGCGAGCTC</td>
</tr>
</tbody>
</table>

<sup>a</sup> Wild-type and M1 to M9 primers are antisense to genomic HDV RNA; the common primer is sense to genomic HDV RNA.

<sup>b</sup> Δ, deletion.
dried and exposed to X-ray film (Kodak XAR). The sizes of protected fragments were compared with that of 32P-labeled dX174 DNA digested with HinfI.

RESULTS

Computer analysis of HDV RNA. (i) Sequence homogeneity within the putative HDV RNA promoter. Previous work from our laboratory (30) identified a bidirectional promoter on HDV cDNA derived from the Italian isolate of HDV (40) that was able to initiate transcription of both genomic and antigenic HDV RNA (Fig. 1). Although this bidirectional promoter activity was restricted to HDV cDNA, the results led to the suggestion that a 29-nt region (nt 1650 to 1679) within the HDV RNA might represent a promoter for RNA-directed RNA transcription. Nucleotide comparison of this region and surrounding sequences between genotype I isolates from Italy (40), the United States (4, 35), Taiwan (9), China (28), Nauru (10), and Lebanon (26) and a genotype II isolate from Japan (19) (Fig. 3) revealed significant stretches of nucleotides with 100% homology. A genotype III isolate (6) showed a similar but not identical structure and was closest to the model of M2 described below (data not shown). The overall homology of the 29-nt region of the Italian isolate compared with the other isolates ranged from 72 to 83% (Fig. 3). Four nucleotides within a GC-rich region (Fig. 3, boxed area) are conserved in all eight HDV isolates examined, indicating a likely functional role of this region, considering the degree of nucleotide divergence in the surrounding nucleotides. A region of 42-nt (nt 1616 to 1658) is conserved in all isolates, although this

FIG. 2. Schematic diagram of the location of HDV RNA transcribed in vitro from plasmids pBHVDV199 (RNA199) and pBM357 (RNA600) in relation to the HDV genome.

putative region of the HDV genome. The 1.7-kb HDV RNA band in each track was selected, and the relative levels of HDV RNA replication in the mutant constructs (pECEM1 to M9) were compared with wild-type (pECE-S/G) levels. In this study, the level of HDV RNA replication directed by these mutants is described as a replication factor compared with levels of wild-type virus RNA replication, which was arbitrarily assigned a value of 1.

Detection of HDAg. HDAg was detected by immunoblot analysis of cell lysates 5 to 6 days posttransfection, using serum from an HDV-infected individual that was adsorbed against normal human liver as described previously (30), and HDAg was visualized by chemiluminescence detection (DuPont, NEN). S-HDAg and L-HDAg standards were obtained from an HDV-infected liver sample. HDAg expression in transfection Cos7 and HeLa cells was confirmed by direct immunofluorescence.

In vitro determination of HDV RNA structure. T7 RNA transcripts were produced from λphl-digested pBHVDV199 which was treated with Klenow DNA polymerase to remove the 3′ overhang prior to RNA synthesis. The DNA template was digested with DNase; the RNA was phenol extracted and then recovered by ethanol precipitation. The RNA was dissolved (1 μg/μl) in renaturation buffer (10 mM Tris·HCl [pH 8.3], 250 mM KCl), heated to 90°C, and allowed to cool gradually to 20°C. For modification by S1 nuclease (Boehringer Mannheim), 1 μl of renatured RNA (1 μg/μl) was digested with increasing concentrations of S1 nuclease (0, 0.1, 0.3, 0.7, and 1 U) in 4 μl of S1 nuclease buffer (30 mM sodium acetate [pH 4.5], 280 mM NaCl, 4.5 mM zinc sulfate, 0.25 μg of RNA per μl) and incubated at 37°C for 5 min. The modified RNA was purified by phenol-chloroform extraction and the RNA was recovered by ethanol precipitation.

Primer extension analysis. Primer extension was used to determine the sites of modification by S1 nuclease digestion and to determine the 5′ end of HDV RNA synthesized in the nuclear extract. The appropriate primers were end labeled with [γ-32P]ATP and T4 polynucleotide kinase. The labeled oligonucleotide (5 ng) was annealed to 1 μg of modified RNA as described above. Primer extension was performed in a 10-μl reaction mixture containing 2 μl of the annealed RNA-labeled primer mix, 1 μl of [32P]ATP to label newly synthesized transcripts. Transcribed RNA was purified by phenol-chloroform extraction followed by ethanol precipitation. The products of the nucleic acid sequencing reactions were separated on a 7% acrylamide–7 M urea gel. The products of the analysis were compared with a DNA sequencing reaction in an adjacent track.

In vitro RNA Pol II transcription. In vitro nuclear transcription assays were performed according to the manufacturer’s protocol, using a HeLa nuclear extract transcription system (Promega). HDV RNAs transcribed in vitro from plasmids pBHVDV199 (RNA199), pBHVDV199M1 to M9, and pBM357 (RNA600) were added to the in vitro nuclear transcription assay mix containing α-32P]UTP to label newly synthesized transcripts. Transcribed RNA was purified by phenol-chloroform extraction followed by ethanol precipitation. The products of the nuclear transcription experiments were separated on a 7% acrylamide–7 M urea gel and visualized by autoradiography.

RNase protection analysis. RNase protection was performed with recombinant RNase One (Promega). 5′-labeled HDV RNA transcribed in the in vitro nuclear transcription assay was recovered by phenol-chloroform extraction, ethanol precipitated, and dried under vacuum. The dried RNA was resuspended in 30 μl of hybridization buffer (80% formamide, 40 mM Na phosphate-NH4-bis[2-ethanesulfonic acid] (PIPES; pH 6.4), 0.4 M sodium acetate, 1 mM EDTA), and 1 μg of probe RNA was added to the mix. The hybridization mix was incubated at 85°C for 3 min and then 37°C for 2 h to allow hybridization of RNA molecules. Three microliters of microfiltered T7 RNA polymerase digestion buffer (10 μM Tris·HCl [pH 7.5], 5 mM EDTA, 200 mM sodium acetate) and 4 μl of RNase One was added and the reaction mix was incubated at 37°C for 1 h. The reaction was stopped by the addition of 10% sodium dodecyl sulfate (SDS) and 4 μg of RNA per μl. Then 825 μl of ice-cold ethanol was added, and the protected RNA hybrids were precipitated overnight at −20°C. The RNA was recovered by centrifugation, washed in 70% ethanol, dried under vacuum, and redissolved in 10 μl of loading dye (80% deionized formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 0.1% SDS). The samples were incubated at 85°C for 5 min prior to loading on a 6% polyacrylamide–7 M urea gel. After separation, the gel was...
region is reduced to 17 nt in the Japanese isolate. No significant homology between this putative promoter region and other known promoter elements was noted with the exception of the GC-rich repeat region of the simian virus 40 (SV40) promoters (41). Thus, this homology search revealed the presence of two highly conserved regions, viz., 4 nt within a GC-rich region and a 17- to 42-nt region (nt 1616 to 1658). These regions are discussed more fully below.

(ii) Computer-predicted RNA secondary structure of the putative HDV RNA promoter. RNA secondary-structure alignments of HDV RNA were performed with the computer program MFOLD version 2.0, which predicts RNA secondary structure by free-energy minimization (20, 21, 43). Although the derived structures represent nt 1601 to 1675, a much larger region of RNA was analyzed (nt 1535 through 1679 to 60) to gain the overall secondary structure of the region.

The secondary structures (Fig. 4) of the eight different isolates were nearly identical. This is not surprising considering the high level of nucleotide homology of this region. However, the Japanese isolate, which has a lower degree of nucleotide homology compared with the other isolates, also showed a very similar secondary structure irrespective of a slight change in the position of the turn of the rod.

The consensus features of the RNA secondary structure, summarized in Fig. 4, comprise a double-stranded lower stem which contains a GC motif (shaded), a conserved internal UCU bulge (arrows), and an upper stem-loop containing an external bulge (asterisks) that precedes the predicted end of the HDV RNA rod. Since these features are conserved this suggests that this region (nt 1608 to 1669) has some functional significance, whereas the structure outside this region is not well conserved between different isolates (data not shown).

Secondary structure of the putative promoter. The above-described model of the secondary structure of the putative promoter region was determined by computer program. To confirm this model, the 199-nt HDV RNA that corresponds to nt 1540 through 1679 to 60 was transcribed in vitro from pBHVDV199, self-annealed, digested with S1 nuclease, and subjected to primer extension analysis.

The S1 nuclease mapping studies were consistent with the predicted RNA secondary structure (Fig. 5A). Increasing concentrations of S1 nuclease revealed a number of prominent bands that were not present in the mock digestion (Fig. 5B). The locations of these bands were determined by comparison with an HDV cDNA sequencing reaction and correspond to the turn in the rod, the external bulge, and the internal UCU bulge. Thus these results confirm the predicted structure of this region.

Identification of an HDV RNA-RNA promoter. The data presented above showed that a region of HDV RNA that corresponds to a cDNA promoter site was highly conserved and had similar structures in different HDV isolates. To determine if this region contained RNA-RNA promoter activity, RNA199, which forms the turn in the rod just upstream of the gene for HDAg, was added to a nuclear extract transcription assay. HDV RNA transcribed from pBM37 (RNA600) that

FIG. 4. Comparison of the predicted HDV RNA secondary structures in the putative promoter regions of different HDV isolates. The GC-rich region which base pairs with the opposite side of the HDV rod structure is enclosed within a shaded box; the conserved internal bulge is indicated by arrows, and the external bulge is represented by asterisks. The references to the isolates are given in the text.

FIG. 5. Common structural features of the putative promoter region as confirmed by enzymatic analysis of HDV RNA synthesized in vitro. (A) Common structural features of the putative promoter regions from the eight different HDV isolates. Three conserved regions are recognized: (i) a GC motif, (ii) a conserved UCU internal bulge, and (iii) a stem loop and bulge region that contains an external bulge. The A residue located at the 5' end of the HDAg mRNA that lies within the external bulge is marked with an asterisk. Arrows indicate regions cleaved by S1 nuclease. (B) A 199-nt HDV RNA transcript was transcribed in vitro and digested with S1 nuclease. Sites of cleavage were determined by primer extension analysis. The left panel represents a DNA sequencing reaction; the right panel represents increasing concentrations of S1 nuclease (0, 0.1, 0.3, 0.7, and 1 U). Regions of specific nucleotide cleavage are indicated.
represented the turn in the opposite end of the rod was added to another reaction as a control. Analysis of the 32P-labeled products of the reaction containing RNA199 by polyacrylamide gel electrophoresis showed two products which were absent in the mock reaction and the reaction which contained RNA600 (Fig. 6). It was considered that these products of approximately 160 and 100 nt could represent genuine HDV RNA transcription or end-labeled products which are often an artifact of the in vitro nuclear extract transcription assay (36). To determine the polarity of the products and thus clarify whether these products resulted from de novo synthesis or end labeling, RNase protection analysis was performed. (The RNase One was previously titrated against single- and double-stranded RNA to determine the optimum concentration [data not shown].) The results of this experiment (Fig. 7) showed that the 100-nt fragment was protected by a genomic probe (RNA199) and thus was antigenomic sense, whereas the larger fragment was not protected by either genomic or antigenomic probes. (This larger fragment was most likely to represent an end-labeled product that was not protected because even after reannealing, the terminal labeled nucleotides derived from the plasmid would still be single stranded.) This experiment was performed three times with the same result. End-labeled products occur only with short RNA molecules (36, 37a), and this probably accounts for the lack of such a product from RNA600.

Thus, RNA199 most likely contains an RNA-RNA promoter. Evidence to support this interpretation was generated in another experiment (see below) using mutants RNA199 that contain changes in the conserved secondary structures in Fig. 4 and 5. Thus, the structure and the motifs of this regions shown in Fig. 4A likely represent an authentic HDV RNA-RNA promoter.

**Primer extension analysis of HDV RNA transcribed in vitro.**

The size of the antigenomic HDV RNA transcribed in the nuclear extract transcription reaction described above was consistent with initiation of transcription close to the turn in the HDV rod. To confirm this possibility and to locate the transcription initiation site, primer extension analysis was performed (Fig. 8). Antigenic HDV RNA, transcribed from RNA199 in the in vitro nuclear extract transcription assay in the absence of radiolabel, was subjected to RNase One protection. This served to remove excess genomic template HDV RNA that may have complicated the primer extension analysis. The protected antigenic HDV RNA product was then subjected to primer extension analysis using primer PE3 (nt 1565 to 1582, CTTCCGGTGTCTTCCTC).

The 5' end of the antigenic RNA product was found to be variable over a span of 20 nt (Fig. 8, track 1), although stronger bands were observed at nt 1618 or 1619 and 1624 or 1625, determined by comparison with a DNA sequencing reaction using the same primer. These bands are likely to represent transcription initiation sites.

To compare the 5' end of the antigenomic HDV RNA product with HDAg mRNA synthesized in vivo, primer extension analysis was performed on poly(A) + RNA (Fig. 8, track 2) isolated from a cell line that constitutively replicates HDV RNA (H169 [31]). The major initiation site for transcription of HDAg mRNA was confirmed at nt 1631, although initiation was variable over a number of nucleotides (nt 1629 to 1634). The variability of initiation is consistent with the in vitro data. Thus, the start site for HDV RNA transcription in the in vitro nuclear extract transcription assay is close to that of HDAg mRNA, within 1 to 13 nt, although they are not exact. The reason for this difference is unknown.

**Mutational analysis of the HDV promoter.** To determine if the major features, i.e., the GC motif and bulge regions, of the promoter region play a role in the initiation of transcription of antigenomic HDV RNA in vivo, we introduced multiple and point mutations into the stem and bulge domains. The exact nucleotide changes are outlined in Table 1, and the effects on the secondary structure of the RNA are depicted in Fig. 9. The effects of these mutations on HDV replication were also examined, and although these results are described below in detail, for convenience the main points of these data are also outlined in context. Consequently, Fig. 9 also shows the relative replication efficiencies of the mutants compared with the wild type.

(i) **GC stem mutations.** The structural effects of mutations within the GC-rich region were examined in M1 (Fig. 9A), in which six consecutive nucleotides within the GC-rich motif were altered, resulting in a loss of the GC stem and other changes to the HDV RNA secondary structure. The result was a complete loss of HDV RNA replication (Fig. 10B, M1). This result confirms that this region is important for HDV RNA replication, although it is possible that it has other roles apart from promoter activity. More subtle mutations (M2 to M4 [Fig. 9A]) which had the effect of altering the GC motif only, leaving the internal UCU bulge and the upper stem-loop and external bulge unchanged, has less effect on HDV RNA replication. The level of replication of M2, in which the GC motif was reduced to four paired nucleotides while retaining the double-stranded nature of this region, was reduced to 0.4 times that of the wild-type construct (Fig. 10B). In contrast, M3 and M4, in which GC motifs of five and six paired nucleotides were retained, respectively, showed levels of replication similar to that of the wild type (Fig. 10B).

(ii) **Stem-loop and bulge region mutations.** To assess the significance of the UCU internal bulge and of the upper stem-loop and external bulge, the structural effects of M5 to M9
HDV replication in cells transfected with HDV cDNA can be initiated from genomic or antigenomic HDV RNA transcribed from a foreign promoter. In this study, 1.3 copies of genomic HDV RNA (transcript 1 in Fig. 1B) were transcribed from plasmid pECE-6C/G from the SV40 early promoter after transfection of cDNA. Processing of this transcript led to initiation of HDV RNA replication. However, transcription from the bidirectional endogenous HDV cDNA promoter is also predicted to result in the synthesis of two additional transcripts of 779 and 1,107 nt (Fig. 1B, transcripts 2 and 3, respectively), although these transcripts were not detected. These predicted subgenomic transcripts are unable to participate in HDV replication, but the 779-nt transcript has the potential to encode S-HDAg (see below). To assess the level of HDV RNA replication initiated by the wild-type (pECE-6C/G) and mutant (pECEtiM1 - M9) HDV constructs, total RNA was purified from HuH7 and Cos7 cells 6 days after transfection and examined for genomic and antigenomic HDV RNA by Northern blot hybridization (Fig. 10). In this experiment, the level of antigenomic HDV RNA is a measure of the relative efficiency of genomic HDV RNA to act as a template for further transcription. Transfection of HuH7 and Cos7 cells gave similar results, but only the results of the Cos7 cell experiments are shown (Fig. 10).

Monomeric, genomic HDV RNA was detected in cells transfected with the wild-type and all mutant constructs (Fig. 10A). This species was probably composed of processed primary transcripts (Fig. 1B, transcript 1) and monomers from further rounds of HDV RNA replication. Monomeric, antigenomic HDV RNA was also detected in cells transfected with the wild type (pECE-6C/G) and M2, M3, M4, and M6 to M9 (Fig. 10B). In contrast, antigenomic HDV RNA was undetected in cells transfected with M1 and M5. Quantification of the levels of genomic and antigenomic HDV RNA was performed by densitometric analyses, and the level of antigenomic HDV RNA was expressed as a replication factor relative to wild-type virus replication, which was arbitrary determined to be 1. The replication factor values (Fig. 9) were consistent in three independent experiments. M1 and M5 showed a complete loss of HDV RNA replication, while M8 and M9 showed an 11-fold reduction in replication. This result suggests that the efficiency of HDV replication was related to the mutations which were introduced into this region.

(ii) Expression of HDAg in transfected cells. The effects of mutations in the HDV RNA promoter region were also examined by the detection of L-HDAg. Since transfection of genomes encoding S-HDAg will result in expression of L-HDAg only after HDV RNA-RNA transcription, the appearance of L-HDAg is an indirect measure of HDV RNA replication. Cos7 and HuH7 cells were transfected with the pECE mutant or wild-type constructs, and cell lysates were prepared 5 to 6 days posttransfection. Immunoblot analysis (Fig. 11) detected similar levels of L-HDAg and S-HDAg in cells transfected with wild-type pECE-6C/G and M2, M3, M4, M6, and M7, respectively. L-HDAg was undetected in cells transfected with M1, M5, M8, and M9.

Thus, these data are in close agreement with the Northern blot hybridization data described above and confirm the replication efficiency of M2, M3, M4, M6, and M7, whereas M1, M5, M8, and M9 were replication defective.

S-HDAg was detected in all cell extracts, including those transfected with replication-defective mutants and most likely reflects expression from transcript 2 (Fig. 1B) synthesized from pECE-6C/G. Thus, the level of S-HDAg in cells transfected with M1, M5, M8, and M9 is a direct measure of expression from the 779-nt primary transcript, and the level in cells trans--
FIG. 9. Comparison of the computer-predicted HDV RNA secondary structures of the wild-type and mutant forms of the HDV RNA promoter. Boxed nucleotides indicate specific nucleotide substitutions or deletions. (A) Mutations introduced to alter the secondary structure of the GC-rich region; (B) mutations to alter the secondary structure of the stem-loop and bulge regions. Levels of HDV RNA replication are expressed relative to a wild-type replication factor of 1. These values represent the averages of three independent experiments as determined by densitometer analysis.
autoradiographic exposure time was 24 h.

...d contains RNA extracted from a stable HDV RNA-positive cell line (H1d). Extracted from mock-transfected Cos7 cells was loaded in track UT, while track M contains RNA extracted from a stable HDV RNA-positive cell line (H1d). Extracted from mock-transfected Cos7 cells...

...since HDV is the only known animal virus with a classical RNA-RNA transcription step that utilizes cellular RNA Pol II.

...promoter for the transcription of antigenomic RNA from genomic RNA. Several lines of evidence are consistent with this interpretation. In particular, (i) this region is located close to the 5' end of the only known mRNA synthesized during HDV replication; (ii) this region is highly conserved between HDV isolates, suggesting that it has an essential function; (iii) this region was extremely sensitive to mutational analysis; and (iv) in general, loss of promoter activity in vitro correlated with loss of HDV RNA replication in vivo. However, our data do not discount the possibility that other HDV RNA promoters are located in the genome, as mutation of this promoter may also affect other unknown functions of that region that are necessary for HDV replication.

...A computer-generated secondary-structure analysis of the promoter revealed a highly ordered RNA secondary structure that constitutes regions of double-stranded RNA with a conserved GC motif, an internal bulge, and a stem-loop and external bulge region that is conserved between different HDV isolates (Fig. 5A). The computer-generated model was confirmed by S1 nuclease mapping and primer extension analysis, and thus the model can be considered authentic.

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DISCUSSION

HDV RNA transcription is unique among animal viruses since HDV is the only known animal virus with a classical RNA-RNA transcription step that utilizes cellular RNA Pol II for transcription (14, 32). We have extended these studies and have now identified and examined the location and structure of the HDV RNA transcriptional promoter. Previous studies determined the location of HDV transcriptional promoters on HDV cDNA (30, 39), but these studies failed to determine if the same region corresponded to a promoter element on HDV RNA. In this present study, a 199-nt genomic RNA molecule containing a region that represents one end of the HDV rod was able to direct the synthesis of antigenomic RNA in an in vitro nuclear extract transcription reaction. Comparison of the sequences of eight HDV isolates showed that a shorter region, nt 1608 to 1669, within this molecule was highly conserved. This region also contains the 29-nt region that corresponds to the bidirectional cDNA promoter. Mutational analysis revealed that this highly conserved region was vital for HDV RNA-RNA transcription in vitro and for HDV replication in vivo, and it is highly likely that this region functions as an RNA promoter for the transcription of antigenomic RNA from genomic RNA. Several lines of evidence are consistent with this interpretation. In particular, (i) this region is located close to the 5' end of the only known mRNA synthesized during HDV replication; (ii) this region is highly conserved between HDV isolates, suggesting that it has an essential function; (iii) this region was extremely sensitive to mutational analysis; and (iv) in general, loss of promoter activity in vitro correlated with loss of HDV RNA replication in vivo. However, our data do not discount the possibility that other HDV RNA promoters are located in the genome, as mutation of this promoter may also affect other unknown functions of that region that are necessary for HDV replication.

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both are necessary for efficient promoter activity. This observation highlights the importance of the secondary structure of this region for HDV RNA replication. Together, these results indicate that the promoter function for HDV RNA replication lies in a region close to the end of the HDV rod that shows conservation of RNA secondary structure between different HDV isolates, in particular the presence of a UCU internal bulge and a stem-loop and external bulge region.

The position of the terminal 5’ nucleotide for HDAd mRNA at position 1631 is noteworthy. This nucleotide, an A residue, is positioned within the internal bulge (AUU) [Fig 5A]) and is located within the potential transcription start site (22, 38). It is unclear if the INR element functions in HDV RNA transcription, although evidence to support its involvement was derived from mutant construct 9 (Fig. 9B) in which the external bulge was transposed to the opposite side of the rod, resulting in a region of double-stranded RNA at the transcription initiation point. This resulted in a 11-fold reduction in HDV RNA replication.

Cellular transcription factors must recognize and bind to the HDV RNA promoter. Other virus RNA molecules with specific structural features are able to bind specific proteins; e.g., the human immunodeficiency virus tat protein interacts with the RNA polymerase II transcription factor TFIID to increase transcriptional efficiency (3,13), and the encapsidation signal of hepatitis B virus RNA is contained in a stem-loop region which interacts with the encapsidation factor HBcAg (22). Cellular proteins have also been shown to interact with viral RNA, as in the case of pyrimidine tract-binding protein, which binds specifically to a bulged hairpin within the internal ribosome entry site of encephalomyocarditis virus and hepatitis C virus (1,17). Thus, the interaction of regions of double-stranded RNA with specific proteins may be more common that is presently recognized.

In contrast to DNA promoter elements, the HDV RNA promoter contains no consensus sequence to bind Pol II transcription factors with the exception of the GC motif (GGGGCC), which is similar to the Sp1 binding site (GGGCGG). It is unclear if this region in HDV RNA can serve as an Sp1 binding site. The HDV RNA promoter also lacks a consensus TATAA-like sequence to specify the direction and site of initiation. However, although most genes contain this motif, a number of genes which do not have been identified (27). The transcription start site from such promoters is less well defined, and in the case of the thymidylate synthase promoter (15,27), initiation can occur within a 90-nt region. Alternatively, the different initiation sites noted between vitro- and in vivo-transcribed HDV RNA may be due to the absence of HDAd, which may play a role in defining the initiation site in vivo. Many promoters which lack a TATA box have bidirectional activity (27). However, we were unable to show that the HDV RNA promoter is bidirectional, because although this study demonstrated promoter activity on genomic HDV RNA for

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