The RNAs of Hepatitis Delta Virus Are Copied by RNA Polymerase II in Nuclear Homogenates

TIE-BO FU AND JOHN TAYLOR*

Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111-2497

Received 8 July 1993/Accepted 29 August 1993

Human hepatitis delta virus has a single-stranded circular RNA genome that replicates by RNA-directed RNA synthesis. The virus encodes only a single protein, the delta antigen, which both is small (22 kDa) and lacks sequence homology to known RNA polymerases, suggesting that the virus employs a cellular polymerase for replication. Consistent with this suggestion, we have used homogenized nuclei from a human hepatoma cell line, HepG2, to demonstrate RNA-directed RNA synthesis from both genomic hepatitis delta virus RNA and its complement, the antigenomic RNA. RNA polymerase II was responsible for this transcription because the reaction was inhibited both by low doses of α -amanitin and by a monoclonal antibody specific for polymerase II. In addition, it was found that the majority of the RNA products were processed, presumably by self-cleavage and self-ligation, to produce covalently closed circular molecules.

Hepatitis delta virus (HDV) is a satellite of human hepatitis B virus with a small single-stranded closed circular RNA genome of about 1,700 nucleotides (7, 19, 22, 23, 39). Replication of viral RNA occurs in the nucleus of an infected cell via RNA-directed RNA synthesis. The template for synthesis of new genomes is a complementary RNA, the antigenome, which is also circular and of unit length. The circularization of genomic and antigenomic RNA is thought to be facilitated by self-cleavage and self-ligation reactions, which have been demonstrated by using purified HDV RNA (21, 31, 32). From studies of cells transfected with a cDNA clone of HDV, it has been possible to initiate replication of the genome in the absence of hepatitis B virus; this replication takes place in many different cell types and is dependent upon the expression of the one protein encoded by HDV, the small form of the delta antigen (5, 20). HDV has many similarities to certain infectious RNA agents of plants, specifically the viroids, virusoids, and satellite RNAs. These similarities include the structure of the RNA; the genomes of the viroids and virusoids are smaller in length than HDV, but like HDV they are circular in conformation and can fold into an unbranched rod-like structure (35). A second similarity is that the RNAs of some of these plant agents are known to undergo self-cleavage and self-ligation (3), just as has been subsequently demonstrated for HDV (21, 31, 32). For the simplest of these plant agents, the viroids, there are reports, including studies with plant cell extracts, that the RNA genomes are transcribed by the plant equivalent of RNA polymerase II (Pol II) (27, 30, 33, 35, 36). This enzyme is thus inferred to act inside an infected cell to replicate the viroid RNA. However, objectively this is only inference; Symons et al. (35) advise caution, especially since other polymerases have been shown to be able to act on a viroid RNA template. For HDV, the only reported reconstruction of RNA-directed RNA synthesis has come from Macnaughton et al. (24).

Macnaughton and colleagues (24) showed that the nuclei of HepG2 cells stably transfected with HDV cDNA produced, in a nuclear run-on reaction, a labeled HDV RNA species in a reaction that could be inhibited by α -amanitin but not by actinomycin D. They also reported similar experiments in

which nuclei were obtained from uninfected HepG2 cells, to which HDV genomic RNA was then added (24). In this second study, nuclei were isolated in the same manner, yet puzzlingly, the authors found that the synthesis was not sensitive to α -amanitin. In neither study were the products of the RNA-directed RNA synthesis characterized electrophoretically, nor was the polarity of the products (genomic versus antigenomic) established.

The experiments described here represent an attempt both to resolve this discrepancy and to extend the results of Macnaughton et al. After modifications of their reaction conditions, it was found that both HDV genomic and antigenomic RNAs could be transcribed, and it was proved that the enzyme involved was RNA Pol II. Furthermore, the HDV RNA products were characterized and found to have undergone specific processing; most of the products were in the form of covalently closed circular molecules, just as observed in natural HDV infections (7, 19, 39). Such data with nuclear homogenates support but do not establish the hypothesis that within an infected cell, the RNA of HDV is replicated by the host RNA Pol II.

MATERIALS AND METHODS

Plasmids and RNA. A full-length cDNA clone of HDV was inserted as a head-to-tail trimer into the transcription plasmid pGem4Z (Promega). The construct so obtained, pGem4Z(D3), was linearized by digestion with *Hind*III or *Bam*HI at a unique site immediately downstream of the HDV insert and then transcribed with the RNA polymerase from phage SP6 (Bethesda Research Laboratories [BRL]) to produce 5.0-kb genomic RNA (22). After synthesis, the reaction mixture was treated with RNase-free DNase I (Promega) prior to extraction as previously described (32). The RNA was analyzed on a gel of 0.8% agarose, in the presence of 10 μ M aurin tricarboxylic acid, and the full-length trimeric transcripts were located by staining with ethidium bromide and then electroeluted (IBI apparatus and procedure).

A specifically deleted form of antigenomic HDV RNA was used as an internal standard in polymerase chain reaction (PCR) assays for viral RNA synthesis. To make this mutant RNA, a pGem4Z construct, pGem4Z(D1) (22), was first deleted between *Hind*III and *Bam*HI in the polylinker region.

* Corresponding author.

Then a deletion was made in the HDV sequence between the sites for *Bst*XI and *Sal*I. To do this, the DNA was first cleaved with *Bst*XI and then blunt ended with T4 DNA polymerase (BRL) (29). Then the DNA was digested with *Sal*I and filled in with the Klenow fragment of *Escherichia coli* DNA Pol I (BRL). The blunt-ended linear DNA was treated with T4 ligase (BRL) to obtain the construct designated pGem4Z(D1ΔBS). This removed 133 bases from the subsequent RNA transcripts.

As HDV templates for DNA-directed RNA synthesis, three HDV cDNA constructs were used. pSVL(D3), as previously described (20), represents the trimeric insert of pGem4Z(D3) transferred to the eukaryotic expression vector pSVL (Pharmacia). Similarly, pCMV(D3) and pCMV(D3r) represent two orientations of the same insert within the vector pCMV, as obtained from Bryan Cullen. Prior to the transcription in nuclear homogenates, these three constructs were cleaved at a unique site immediately 3' of the cDNA insert. For the studies with potato spindle tuber viroid (PSTV), a dimeric cDNA clone, pST64-B14, was obtained from Robert Owens (26). This insert was transferred to pGem4Z for transcription of plus- and minus-strand PSTV RNAs by the phage polymerases. It was also transferred to the eukaryotic expression vector pMT, utilizing the metallothionein promoter, for DNA-directed transcription by nuclear homogenates. In all cases, the constructs were cleaved 3' of the insert prior to RNA transcription.

Nuclear homogenates. Monolayer cultures of HepG2 (1), HeLa, Huh7 (25), and Cos7(Ag) (12) cells were used as sources of nuclei following a modification of the procedure of Macnaughton et al. (24). At 2 days after seeding, monolayer cultures containing about 5×10^7 cells were washed twice with ice-cold phosphate-buffered saline and then overlaid with lysis buffer (3 mM MgCl₂, 1 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM Tris-HCl [pH 7.4], 0.5% Nonidet P-40) at 4°C for 10 min. The disrupted cells were then scraped from the dish, layered over 30% sucrose in lysis buffer lacking Nonidet P-40, and centrifuged at $500 \times g$ for 10 min. The nuclear pellet was recovered and washed twice with storage buffer (40% glycerol, 5 mM MgCl₂, 0.1 mM EDTA, 50 mM Tris-HCl [pH 8.3]). After resuspension in 0.5 ml of this buffer, the nuclei were disrupted by 40 strokes in a Dounce homogenizer, and then aliquots were stored at -70°C .

The standard RNA synthesis reaction mixture contained 10 μl of HepG2 homogenate added to 15 μl of reaction buffer containing 2.5 mM MgCl₂, 40 mM KCl, 17 mM dithiothreitol, 250 U of RNasin (Promega) per ml, and four ribonucleoside triphosphates (rNTPs), each at 0.67 mM. (Note that the final concentration of KCl was 25 mM; preliminary experiments showed that 0, 12.5, and 25 mM KCl were superior in RNA transcription to 50 and 100 mM.) Typically 100 ng of DNA or RNA template was added. After 60 min at 30°C, the reaction mixture was treated with sodium dodecyl sulfate and pronase prior to extractions, once with phenol, and then twice with ether. Samples were collected by precipitation with ethanol in the presence of salt and carrier dextran (32). Following transcription from DNA templates, the isolated nucleic acids were digested with RNase-free DNase I (Promega) and then subjected to the same extraction procedure.

For the antibody inhibition studies represented in Table 2, we used a mouse monoclonal antibody, designated 8WG16, of class immunoglobulin G2a, that binds to the C-terminal domain of wheat germ RNA Pol II (Promega) (37) and a rabbit polyclonal antibody reactive with mouse IgG (Sigma). For some of these studies, the antibodies were combined and

incubated for 30 min at room temperature prior to being added to the reaction mixtures.

Quantitative nested RNA-PCR. The basic strategy was as described by Gilliland et al. (10). The sequences of the four primers in relation to the HDV genome sequence (22) are as follows: P, 701-AGCCTCCTCGCTGGCGCCGGCTGGGCAACATTCCG-735; Q, 1217-GAGGAAGTCAAGGAGGTTGACCGAGGAAGACGAGAG-1183; R, 737-GGGGACCGTC CCTCGGTAATGGCGAATGGGACCC-771; and S, 1152-TGGGGGTGTGAACCCCTCGAAGGTGGATCGAGG G-1118. To detect antigenomic RNA, primer P was used in an initial reverse transcription. (To detect genomic RNA, primer Q was used.) The reverse transcriptase was from avian myeloblastosis virus (Life Sciences, St. Petersburg, Fla.), and the template was 10% of the nuclear homogenate reaction along with a fixed amount of specifically deleted HDV antigenomic RNA to act as an internal standard. The product was extracted, and 10% was used as the input for the first PCR using primers P and Q. Then 10% of this product was used as template for the second PCR using primer S and end-labeled primer R. Reaction mixes of 10 μl containing 0.3 U of Vent polymerase (New England Biolabs) were heat sealed in microdispenser tubes (Drummond). PCRs of 35 cycles (denaturation for 5 s at 94°C, annealing for 5 s at 55°C, and elongation for 15 s at 72°C) were carried out in less than 30 min, using an Air Thermo-Cycler (Idaho Technologies). The labeled PCR products were extracted and denatured with glyoxal prior to analysis on gels of 2.5% agarose. The denaturing conditions were necessary to remove mixed hybrids between full-length and deleted PCR products (10). After electrophoresis, the gels were dried onto DE-81 paper (Whatman) and subjected to autoradiography and/or direct quantitation with a radioanalytic imaging system (AMBIS, San Diego, Calif.).

Depending on the choice of primer used in the reverse transcriptase reaction, this assay was able to quantitate both genomic and antigenomic HDV RNA, using the corresponding strand of the internal standard. The assay for antigenomic RNA spans the site at 900/901 where self-cleavage can occur (32). Thus, the assay could detect only molecules that had never self-cleaved and those that had both self-cleaved and self-ligated.

Northern (RNA) analyses. RNA samples were glyoxalated and analyzed on horizontal agarose gels as previously described (7). Three special conditions were used to resolve linear and circular conformations. First, the agarose gel concentration was raised to 3%. Second, the gel combs were reduced to 0.5-mm thickness. Third, a much longer electrophoresis time was used, specifically, 6 h at 5.5 V/cm.

RESULTS

HDV RNA transcription conditions assayed by quantitative PCR. For the proposed studies of transcription of HDV RNAs, it was essential to have a quantitative assay of the RNA products. An adaption of the procedure of Gilliland et al. (10) was used to assay for either genomic or antigenomic HDV RNA. The nested RNA-PCR was carried out with an internal standard, a fixed amount of a deleted HDV RNA that should create a labeled product of 283 bases. In contrast, the unmodified HDV RNA should create a species of 416 bases. In a reconstruction experiment in which the assay was for antigenomic RNA, a range of amounts of the unknown was quantitated in the presence of a fixed amount of internal standard. A comparison was made of the predicted ratios of unknown to internal standard, based on the optical density of the input RNAs, and the observed ratios, based on direct

FIG. 1. Variables affecting RNA-directed transcription of HDV RNA. A series of assays was set up and subsequently quantitated by the RNA-PCR assay. The standard reaction of 60 min at 30°C was modified as follows: lane 1, 0 min; lane 2, standard reaction; lane 3, minus RNA template; lane 4, minus rNTPs; lane 5, plus α -amanitin (1 μ g/ml); lane 6, plus actinomycin (20 μ g/ml); lane 7, plus DNase I (50 μ g/ml); lane 8, plus SLS (0.025%); lane 9, as in lane 8 but added at 15 min; and lane 10, with homogenate preheated for 10 min at 60°C. In the RNA-PCR assay, we used 0.1 ng of internal standard and an aliquot of the extracted product containing 10 ng of genomic RNA. Direct quantitation of unknown relative to the internal standard was used to deduce the indicated ratio observed.

quantitation of the gel analysis of the products. The two values were in close agreement (within a factor of 2) around the regions of equivalence (9).

This assay was used to quantitate RNA-directed RNA transcription achieved by nuclear homogenates of HepG2 cells (1), prepared by a modification of the procedure of Macnaughton et al. (24). Head-to-tail trimers of HDV genomic RNA were added as the template, and the standard reaction was carried out for 60 min at 30°C, after which the quantitative nested RNA-PCR assay was used to measure the synthesis of antigenomic HDV RNA. In most assays, the amount of internal standard was chosen to correspond to 1% of the amount of template RNA. Thus, if the observed ratio for the unknown relative to the internal standard was 2.0, then within the linearity of the quantitative PCR assay, it could be deduced that about 2.0% of the genomic RNA template was copied into antigenomic RNA.

Figure 1 shows the autoradiogram for 10 such assays along with quantitation obtained by using the internal standard. Note that the synthesis of antigenomic RNA was time dependent. None was detectable at 0 min (lane 1), and the level increased during 60 min to about 1% (lane 2). No synthesis was detected in the absence of template (lane 3) or in the absence of rNTPs (lane 4). The reaction was inhibited by α -amanitin present at 1 μ g/ml (lane 5). Such inhibition is consistent with transcription by RNA Pol II (2). The reaction was not significantly inhibited by either actinomycin (lane 6) or RNase-free DNase I (lane 7). The actinomycin concentration (20 μ g/ml) was sufficient to inhibit DNA-directed RNA synthesis by Pol II (2). The DNase I concentration (50 μ g/ml) was separately shown to be sufficient to destroy a labeled DNA added to the reaction mixture (9). Others have shown that 0.025% sodium lauryl sarcosinate (SLS), when added to DNA-directed reactions, can block the initiation of Pol II transcription (13). This also was true for the

TABLE 1. Variables affecting transcription reaction

Expt	Conditions	Observed ratio ^a
1	Reaction times (min)	
	0	<0.01
	15	<0.01
	30	1.38
	60 ^b	1.59
	120	1.71
	240	0.76
2	Reaction temp (°C)	
	25	0.47
	27.5	0.76
	30 ^b	1.56
	32.5	0.96
3	Antigenomic RNA template ^c	0.96
4	Sources of cells for nuclear homogenate ^d	
	HepG2 ^b	1.01
	Cos7(Ag)	1.19
	HeLa	0.31
	Huh7	0.18

^a Deduced from RNA-PCR as described for Fig. 1.

^b Corresponds to the standard reaction conditions.

^c To assay genomic RNA, the assay was modified as described in Materials and Methods.

^d Nuclear homogenates were prepared as described in Materials and Methods. Cos7(Ag) cells are a line of Cos7 cells (12) that have been stably transfected with a construct expressing the small form of the delta antigen.

RNA-directed synthesis of antigenomic RNA (lane 8). However, such transcription can become resistant to SLS and is indicative of the formation on the template of an initiation complex (16, 34). Apparently, a similar phenomenon occurred also for RNA-directed RNA synthesis because when SLS was added at 15 min, no effect on transcription accumulated by 60 min was observed (lane 9). Finally, if the homogenate was heated at 60°C for 10 min prior to use, no synthesis could be detected (lane 10).

More details of the transcription reaction are summarized in Table 1. The time dependence of the reaction was such that no detectable synthesis occurred in the first 15 min. This was the time at which addition of SLS caused no blockage to the accumulation achieved by 60 min (Fig. 1, lane 9). The reaction was also temperature dependent, with an optimum around 30°C. When the genomic RNA template was replaced with antigenomic RNA, transcription into genomic RNA was observed. The nuclear homogenate from HepG2 cells was also replaceable by similar homogenates made with a monkey kidney cell line (12), a human cervical cell line, and another line of human liver cells (25). Not shown are negative results obtained with homogenates made from *Drosophila* embryos (9).

Template requirements for transcription as assayed by Northern analysis. The foregoing studies quantitated only the transcription of HDV genomic and antigenomic RNA. To examine the sizes of these products and also to test templates other than HDV RNA, use was made of Northern analysis. The aims included an examination of RNA synthesis relative to DNA-directed RNA synthesis and also an examination of RNA templates other than HDV.

For the first study, the RNA transcription products were glyoxalated and examined by electrophoresis into gels of 0.7% agarose, followed by Northern analysis for both genomic RNA (Fig. 2A) and antigenomic RNA (Fig. 2B). Both DNA size

FIG. 2. Northern analysis of RNA- and DNA-directed transcription of HDV RNAs. Nuclear homogenates were used to transcribe HDV RNA and DNA templates. Aliquots of the extracted products were glyoxalated and analyzed on gels of 0.7% agarose. After electrotransfer, the filters were hybridized to detect genomic RNA (A) or antigenomic RNA (B). Lane 1, end-labeled *Hind*III fragments of phage lambda DNA; lanes 2 and 3, 0.1 and 1 ng of 1.8-kb antigenomic HDV RNA standard; lanes 16 and 17, 0.1 and 1 ng of 1.8-kb genomic HDV RNA standard. Lanes 4 to 15 show RNA products after 0- and 60-min reactions for the following HDV templates: lanes 4 and 5, no template; lanes 6 and 7, trimers of genomic RNA; lanes 8 and 9, trimers of antigenomic RNA; lanes 10 and 11, pSVL(D3); lanes 12 and 13, pCMV(D3r); and lanes 14 and 15, pCMV(D3).

markers (lane 1) and known amounts of 1.8-kb RNA standards of antigenomic RNA (lanes 2 and 3) and genomic RNA (lanes 16 and 17) were used. Control reactions without added template showed no bands at either 0 or 60 min (lanes 4 and 5).

Transcription from 5.0-kb trimers of HDV genomic RNA was tested at 0 min (lane 6) and 60 min (lane 7); the reactions contained no antigenomic RNA at 0 min (lane 6), but during the 60-min reaction there appeared a major species of about 1.7 kb (Fig. 2B, lane 7). Similarly, with trimers of antigenomic RNA as the template (Fig. 2A, lanes 8 and 9), the time-dependent appearance of 1.7-kb genomic RNA product was observed.

As a control for these studies, the transcription from HDV cDNA constructs as directed by known RNA Pol II promoters was examined in parallel. The first construct, pSVL(D3), contained a trimer of HDV cDNA, in which the simian virus 40 late promoter drives the transcription of genomic HDV RNA. This construct was cleaved immediately downstream of the HDV sequences prior to addition of the nuclear homogenate. A time-dependent appearance of a 1.7-kb genomic RNA product (Fig. 2A, lanes 10 and 11) was observed. A second DNA-directed transcription was carried out with another precleaved construct, this time with pCMV(D3), a trimer of HDV cDNA downstream of the cytomegalovirus (CMV) immediate-early promoter. Again there was a time-dependent appearance of 1.7-kb genomic RNA (Fig. 2A, lanes 14 and 15). A third construct, pCMV(D3r), with the precleaved cDNA in the reverse orientation relative to the CMV promoter, produced not genomic but antigenomic RNA, again in a time-dependent manner (Fig. 2B, lanes 12 and 13).

These studies showed that the nuclear homogenates were

FIG. 3. Northern analysis of RNA- and DNA-directed transcription of PSTV RNAs. Nuclear homogenates were used to transcribe PSTV RNA and DNA templates. Aliquots of the extracted products were glyoxalated and analyzed on gels of 0.7% agarose. After electrotransfer, the filters were hybridized to detect minus-strand RNA. Lane 1, end-labeled *Hind*III fragments of phage lambda DNA; lane 2, 1 ng of 0.718-kb plus-strand RNA standard; lane 3, 1 ng of 0.718-kb minus-strand RNA standard; lanes 4 and 5, 0.718-kb plus-strand RNA template incubated for 0 and 60 min in nuclear homogenate. In lanes 6 and 7, a 0.718-kb cDNA construct was similarly incubated for 0 and 60 min in nuclear homogenate; this construct contain a dimer of PSTV cDNA downstream of the metallothionein Pol II promoter and oriented so as to expect transcription of minus-strand RNA.

able to carry out DNA-directed RNA synthesis from DNA templates by using known Pol II promoters as well as carry out RNA-directed RNA synthesis from both genomic and antigenomic HDV RNAs. Direct quantitation of the Northern analyses showed that the efficiencies of the nuclear homogenate for the five different RNA synthesis reactions were roughly comparable, even within a factor of 2.

It was intriguing that all of these various HDV RNA products migrated as discrete species of 1.7 kb. A preliminary interpretation was that either at or after transcription, the RNAs were undergoing self-cleavage by using the domain that is known to be present once per unit length (21, 32). As will be discussed below, this interpretation was confirmed and extended.

A second series of experiments was then carried out to determine whether RNA species other than HDV could be transcribed by the nuclear homogenates. The template tested was a 0.718-kb head-to-tail dimer of the so-called plus-strand RNA of PSTV. As before, a Northern analysis of the RNA transcription products was carried out. As shown in Fig. 3, no time-dependent appearance of minus-strand RNA was detected (lanes 4 and 5). As controls for this, 0.718-kb plus and minus strands as synthesized by using phage polymerases (lanes 2 and 3, respectively) were used. As an additional positive control, a dimer of PSTV cDNA under the control of the Pol II promoter for metallothionein was used; this template did produce a 0.718-kb minus-strand RNA in a time-dependent manner (lanes 6 and 7). It was thus concluded that PSTV RNA, even though it has the potential to fold into a rod-like structure analogous to HDV RNA, was nevertheless unable to act as a template for RNA-directed RNA synthesis by the nuclear homogenates. Since others have reported that the RNA Pol II from plants can copy PSTV (30, 33), it would seem that the corresponding mammalian enzyme will not substitute.

Conformation of HDV RNA transcripts. The foregoing studies indicated that in both RNA- and DNA-directed transcription of HDV sequences, the RNA products were primarily of 1.7 kb. In the HDV-infected liver and in purified virions, the

FIG. 4. Northern analyses of RNA-directed transcription of HDV RNA in relationship to HDV RNAs from infected liver. Nuclear homogenates were used to transcribe genomic RNA as in Fig. 1, lanes 1 and 2. Aliquots of the extracted product were glyoxalated and analyzed on either a 0.8% agarose gel (A and B) or a 3% gel (C). After electrotransfer, the filters in panels A and C were hybridized to detect antigenomic RNA. The filter of panel A was subsequently stripped and rehybridized to detect genomic RNA as in panel B. Lanes for all panels: 1, end-labeled *Hind*III fragments of phage lambda DNA; 2, 1 ng of 1.8-kb antigenomic HDV RNA standard; 3, standard reaction at 0 min; 4, standard reaction; 5, premix of lanes 4 and 6; 6, 20 ng of total RNA from the liver of an infected woodchuck; 7, 1 ng of 1.8-kb genomic HDV RNA standard. For lanes 3 to 5, the amount of genomic RNA corresponded to 20, 20, and 50 ng of template for panels A to C, respectively. The autoradiogram exposure times were 15 (A), 3 (B), and 15 (C) h. In lanes 3 to 5 of panel A, there are bands at about 5.0 and 6.5 kb. The presence of these bands was independent of reaction time. Both bands represent cross-reaction of the probe with RNA species present in the homogenate alone, as they can also be seen in lanes 4 and 5 of Fig. 3.

major species is also 1.7 kb in length (7). Also, these RNAs exist in two conformations: as a linear species and as an excess of circular species (7, 19, 39). Thus, a more extensive Northern analysis was undertaken to more carefully examine the products of transcription by nuclear homogenates and to compare and contrast these with the HDV RNAs from an infected liver.

Figure 4 shows three Northern analyses. Lanes 3 and 4 represent the RNAs extracted from a reaction containing a genomic HDV trimeric RNA template after 0 and 60 min, respectively, lanes 6 are samples from the liver of an HDV-infected woodchuck, and lanes 5 are mixtures of the samples in lanes 4 and 6.

Figure 4A shows a 0.8% gel analysis to detect antigenomic RNA. Clearly the nuclear homogenate produced a predominant species (lane 4) that not only was similar to the liver sample (lane 6) but actually comigrated with it (lane 5). Relative to the DNA markers (lane 1) and the 1.8-kb antigenomic RNA standard (lane 3), this major species behaved as expected for 1.7-kb unit-length HDV RNA. As a control for this analysis, the filter was rehybridized to detect HDV genomic RNA. As shown in Fig. 4B, we then detected the template RNA (lanes 3 to 5) and a 1.8-kb genomic RNA standard (lane 7). It can be seen that the 5.0-kb genomic RNA template did not undergo significant degradation during the 60-min transcription reaction. It might seem that much of the template RNA was degraded even prior to transcription (lane 3). This is considered deceptive in that the transfer efficiency was lower for higher-molecular-weight species (lane 1); these end-labeled fragments of phage lambda DNA were originally present in equimolar amounts, and yet the larger fragments were transferred with a lower efficiency. However, since the yield of product as measured by PCR was $\leq 2\%$, it is not excluded that species smaller than the 5.0-kb genomic RNA acted as a template for the observed RNA-directed transcription.

Figure 4C shows a similar gel analysis for antigenomic RNA, but this time with 3% agarose, so as to separate species of the same length but with different conformation (9). As shown in Fig. 4C, this separated the product RNA into two species (lane

4). The RNA from infected liver also separated into the same two major species (lane 6), the identity of which was confirmed by premixing the two samples (lane 5). The less abundant species migrated as if it were 1.7 kb. Note that it migrated faster than the 1.8-kb linear RNA standard (lane 2). On the basis of these mobilities and previous experience (7), the faster species was designated as linears and the slower species was designated as circles. Quantitation showed that this putative circular species was about four times more abundant than the linear species. Apparently for the reaction, just as the *in vivo* situation, the majority of the surviving antigenomic RNA was that which was processed to a circular form.

Previous studies have shown that the genomic and antigenomic HDV RNAs each contain one site per unit length at which specific self-cleavage can occur (21, 32). Thus, the product species that were 1.7 kb may have arisen from larger transcripts that were processed at least twice. From Fig. 4B, it would seem that such processing was not occurring on the preformed genomic trimers that were added to the nuclear homogenates. Apparently the RNA processing in the nuclear homogenates was largely coupled with Pol II-dependent transcription.

Additional evidence that transcription was via RNA Pol II. The quantitative RNA-PCR studies presented in Table 1 provided strong evidence that the transcription was from an RNA template. The inhibitory activity of α -amanitin was the only evidence that the enzyme involved was RNA Pol II. To directly test this interpretation, use was made of a mouse monoclonal antibody specific for the heptad repeat in the C-terminal domain of the large subunit of RNA Pol II (37). As shown in Table 2, experiment 1, the standard reaction was inhibited by the addition of this antibody. As a control for nonspecific effects, this antibody was preincubated with increasing amounts of a second antibody, a rabbit polyclonal antibody specific for mouse immunoglobulins. Increasing amounts of this second antibody relieved the inhibition caused by the Pol II-specific antibody. As a control, the rabbit antibody alone, even in the highest dose, had no effect on transcription.

TABLE 2. Inhibition of transcription reaction by antibody specific for RNA Pol II

Expt	Conditions	Observed ratio ^a
1	Antibodies added to homogenate prior to standard reaction ^b	
	No additions	2.54
	Mouse anti-Pol II (1.4 µg)	<0.02
	Mouse anti-Pol II (1.4 µg) and rabbit anti-mouse Ig (0.3 µg)	<0.03
	Mouse anti-Pol II (1.4 µg) and rabbit anti-mouse Ig (1.4 µg)	<0.02
	Mouse anti-Pol II (1.4 µg) and rabbit anti-mouse Ig (7 µg)	1.52
2	Rabbit anti-mouse Ig (7 µg)	2.19
	Anti-Pol II antibody (1.4 µg) added during 60-min standard reaction	
	No additions	1.43
	Added at 0 min	<0.01
3	Added at 15 min	0.33
	Anti-Pol II antibody (1.4 µg) added during 60-min DNA-directed reaction ^c	
	No additions	4.43
	Added at 0 min	0.11
	Added at 15 min	0.89

^a Deduced from RNA-PCR as described for Fig. 1. In all RNA-PCR assays, 0.1 ng of internal standard and an aliquot of the extracted product containing 25 ng of genomic RNA were used.

^b The aliquot of nuclear homogenate and indicated antibodies were preincubated as described in Materials and Methods and then added to the standard 60-min reaction.

^c The standard reaction was modified by replacing the RNA template with an equal amount of the DNA construct cleaved just downstream of pCMV(D3r), a trimer of HDV cDNA under the control of the CMV promoter, so as to direct the transcription of antigenomic RNA.

An alternative experimental approach was achieved by adding the monoclonal antibody at or during the standard reaction. As shown in Table 2, experiment 2, this produced a time-dependent inhibition of the RNA-directed RNA synthesis. A parallel study was carried out for the DNA-directed RNA synthesis from the pCMV(D3r) template, which was expected to be via Pol II, under the control of the CMV immediate-early promoter. As shown in Table 2, experiment 3, a comparable time-dependent inhibition was obtained.

Together, these data provide strong evidence that the majority of the RNA- and DNA-directed RNA transcription detected with the nuclear homogenates involved RNA Pol II. It should be noted that inhibition of Pol II by the mouse monoclonal antibody was probably facilitated by two things: first, there are on the large subunit of mammalian Pol II around 52 copies of the heptad repeat to which the monoclonal antibody reacts (34), and second, there is evidence that the carboxy-terminal domain is specifically needed for an intercalation with the template (34).

DISCUSSION

The evidence presented here indicates that HDV genomic and antigenomic RNAs were copied in nuclear homogenates by RNA Pol II. It cannot be inferred that other polymerases in the nuclear homogenate, Pol I and Pol III, are incapable of such transcription, because it was not shown that they were otherwise active in the homogenate. Most of the studies presented used homogenates from HepG2 cells, a line of human hepatoma cells (1). However, similar results were seen with another liver cell line, Huh7 (25), and with the nonliver cell lines HeLa and Cos7(Ag) (12) (Table 1). In contrast, a nuclear homogenate of *Drosophila* embryos (Stratagene) failed to work (9). Also tested without success were the purified RNA polymerases of phages SP6 and T7 (9); these polymerases were tested because of reports by Konarska and Sharp (17, 18), which showed that RNA-directed RNA synthesis could be achieved with phage T7 polymerase acting on a specific RNA template.

The quantitation of the HDV RNA-directed RNA synthesis

obtained with the nuclear homogenates revealed yields of 0.5 to 2% relative to the amount of template RNA. Within a factor of 2, a similar efficiency was obtained for DNA-directed transcription using known Pol II promoters (Fig. 2). Such yields were comparable to those reported by others for RNA Pol II synthesis in nuclear homogenates as directed by DNA templates (16).

The HDV RNAs, as synthesized by the nuclear homogenates, were multiply processed. This was true for DNA- and RNA-directed transcription and for both genomic and antigenomic products (Figs. 2 and 4) (9). As shown in Fig. 4C, two conformations of unit-length RNA product were identified: both linear and an excess of circles. The circular species presumably arose via RNA transcripts that underwent at least two self-cleavages and one self-ligation. (Remember that the HDV RNA and DNA templates used for transcription were all head-to-tail linear trimers of the 1.7-kb HDV sequence. Similar results were, however, obtained in preliminary studies with circular genomic monomers as obtained from virions [9].) In some ways, a precedent for such multiple processing of RNA transcripts was set by the studies of Buzayan et al. with the satellite RNA of tobacco ringspot virus (3). They made transcripts off DNA with a phage polymerase and obtained similar multiply processed transcripts. With HDV, the yield of circles relative to linears was much higher than that obtained by Buzayan et al. (3). This could reflect more efficient processing of HDV RNAs. It should be noted that Tsagris et al. (38) have found that multimeric linear RNAs of a plant viroid are processed to monomeric covalently closed circles in the presence of RNase T₁. The possibility of such nuclease-driven processing is difficult to exclude, even though no significant change in the electrophoretic mobility of template RNAs could be detected after a 60-min reaction (Fig. 2 and 4B). It should also be noted that plant RNA Pol II will transcribe the plant viroid RNAs, and there are conflicting reports, both for and against the ability of such RNA products to undergo subsequent processing (27, 30, 33, 35, 36).

The initiation sites of HDV RNA-directed RNA synthesis were not readily apparent from the Northern analyses (Fig. 2

and 4), nor were they detected by preliminary primer extension assays (9). One explanation of this absence might be that HDV RNA-directed transcription was achieved by the RNA templates also acting as primers, for example, via some kind of 3'-terminal hairpin structure. Such a priming would change the electrophoretic mobility of the template RNA. This was not detected (Fig. 4B) but could have been missed, since the yield was <2% (Table 1) and also because the products underwent self-cleavage. The inability to detect the initiation sites should be compared with studies of HDV RNAs as synthesized in infected liver and transfected cultured cells (7, 20). Such studies are in fact quite similar in that they also have not yet revealed specific sites for the initiation of genomic RNA. However, for antigenomic RNA, relatively small amounts of an 800-base polyadenylated species were sometimes, but not always, seen in Northern analyses (7, 14, 15). (Note that in the current analysis of antigenomic RNA from an HDV-infected liver [Fig. 4A and C, it was not readily detected either.] This 800-base species, the putative mRNA for the delta antigen, has been characterized and found by primer extension to have a 5'-end 5 bases from the top of the rod-like HDV RNA structure (14).

Further studies are thus needed to clarify the initiation sites for HDV RNA transcription both as it occurs inside infected cells and as it occurs with nuclear homogenates. It remains possible, as Diener has proposed for viroids (8), that there is not an obligatory need for specific initiation sites, since the nascent RNAs can undergo posttranscriptional processing. It is also possible that HDV differs from the viroids in that the delta antigen might have a role in creating specific initiation sites; the delta antigen will specifically recognize the HDV rod-like structure (6), and it is possible that in so doing, many potential initiation sites are blocked, leaving behind a much smaller subset of sites. Such a hypothesis can now be tested in nuclear homogenates, since ribonucleoprotein structures composed of delta antigen and HDV RNA have been isolated from virions and from the nuclei of infected cells (28).

The transcription studies of Macnaughton et al. (24) and most of the studies presented here occurred in the absence of the delta antigen. However, with nuclear homogenates from Cos7(Ag), a Cos7 cell line stably transfected and expressing the small form of the delta antigen, no major change in the ability to transcribe added HDV RNA was detected (Table 1). Such findings will have to be reconciled with what happens inside cells; in transfection studies with HDV cDNA or cRNA, there is no replication of the HDV genome unless the small form of the delta antigen is present (11, 20).

The efficiencies of transcription of antigenomic RNA relative to genomic RNA were comparable (Table 1 and Fig. 2). At first glance, a significant difference could have been expected, since inside an infected cell there is usually 10 times more genomic than antigenomic RNA (7). More reasonably, it would appear that inside the infected cell, additional variables affect the ratio of genomic to antigenomic RNA; an obvious variable is that antigenomic RNA transcripts differ from genomic in that they can undergo polyadenylation (7, 14).

As shown in Fig. 3, a non-HDV RNA, that from PSTV, was not transcribed by the nuclear homogenates. This negative result testifies to the specificity of the observed transcription of HDV RNA; the data are thus not analogous to the report that the purified bacterial DNA-directed RNA polymerase can promiscuously copy RNA templates (4). At the same time, this particular negative result with PSTV was surprising in that viroids, like HDV RNA, have the ability to fold into the rod-like structure (35). Also, this particular viroid has been reported by others to be transcribed by Pol II in homogenates

from plant cells (27, 30, 33, 36). It remains possible that there is a critical difference between the RNA Pol II species from plants versus animals in the ability to accept a plant viroid RNA as a template.

Two aspects of this study indicate that HDV RNA-directed RNA synthesis might be analogous to DNA-directed RNA synthesis in that some form of initiation complex has to be assembled on the template (16, 40). Specifically, there was a lag of at least 15 min before the appearance of RNA-directed products detectable by RNA-PCR (Table 1), and if SLS, a known inhibitor of the formation of DNA-directed initiation complexes (16), was added prior to this 15 min, subsequent transcription was blocked (Fig. 1) (9). Further studies are now needed to test this interpretation and to compare and contrast the transcription factors that are involved. At the same time, an investigation needs to be made of the *cis*-acting sequences and/or structures that are required on the RNA template.

ACKNOWLEDGMENTS

J.T. was supported by grant VM-58R from the American Cancer Society, grants CA-06927 and AI-26522 from the National Institutes for Health, and an appropriation from the Commonwealth of Pennsylvania.

We thank Tony Yeung for the chemical synthesis of the oligodeoxynucleotide primers and Robert Owens and Bryan Cullen for providing plasmids. Valuable advice was given by David Lazinski and Roberto Weinmann. Bill Mason, David Lazinski, Christoph Seeger, and Hans J. Netter provided constructive criticism of the manuscript.

REFERENCES

1. Aden, D. P., A. Fogel, S. Plotkin, I. Damjanov, and B. B. Knowles. 1979. Controlled synthesis of HBsAg in differentiated human liver carcinoma-derived cell-line. *Nature (London)* **282**:615-616.
2. Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. Molecular biology of the cell. Garland Publishing Co., New York.
3. Buzayan, J. M., W. L. Gerlach, and G. Bruening. 1986. Non-enzymatic cleavage and ligation of RNAs complementary to a plant satellite RNA. *Nature (London)* **323**:349-353.
4. Chamberlin, M. J. 1974. Bacterial DNA-dependent RNA polymerase, p. 333-374. In P. Boyer (ed.), *The enzymes*, vol. 10. Academic Press, New York.
5. Chao, M. 1991. Ph.D. thesis. University of Pennsylvania, Philadelphia.
6. Chao, M., S.-Y. Hsieh, and J. Taylor. 1991. The antigen of hepatitis delta virus: examination of in vitro RNA-binding specificity. *J. Virol.* **65**:4057-4062.
7. Chen, P.-J., G. Kalpana, J. Goldberg, W. Mason, B. Werner, J. Gerin, and J. Taylor. 1986. Structure and replication of the genome of hepatitis delta virus. *Proc. Natl. Acad. Sci. USA* **83**:8774-8778.
8. Diener, T. O. 1989. Circular RNAs: relics of precellular evolution? *Proc. Natl. Acad. Sci. USA* **86**:9370-9374.
9. Fu, T.-B., and J. M. Taylor. Unpublished observations.
10. Gilliland, G., S. Perrin, and H. F. Bunn. 1990. Competitive PCR for quantitation of mRNA, p. 60-75. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), *PCR protocols*. Academic Press, San Diego, Calif.
11. Glenn, J. S., J. M. Taylor, and J. M. White. 1990. In vitro-synthesized hepatitis delta virus RNA initiates genome replication in cultured cells. *J. Virol.* **64**:3104-3107.
12. Gluzman, Y. 1981. SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**:175-182.
13. Hawley, D. K., and R. G. Roeder. 1987. Functional steps in transcription initiation and reinitiation from the major late promoter in a HeLa nuclear extract. *J. Biol. Chem.* **262**:3452-3461.
14. 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.
15. Hsieh, S.-Y., and J. Taylor. 1991. Regulation of polyadenylation of

- hepatitis delta virus antigenomic RNA. *J. Virol.* **65**:6438–6446.
16. **Kadonaga, J. T.** 1989. Assembly and disassembly of the *Drosophila* RNA polymerase II complex during transcription. *J. Biol. Chem.* **265**:2624–2631.
17. **Konarska, M. M., and P. A. Sharp.** 1989. Replication of RNA by the DNA-dependent RNA polymerase of phage T7. *Cell* **57**:423–431.
18. **Konarska, M. M., and P. A. Sharp.** 1990. Structure of RNAs replicated by the DNA-dependent T7 RNA polymerase. *Cell* **63**:609–618.
19. **Kos, A., R. Dijkema, A. C. Arnberg, P. H. van der Meide, and H. Schellekens.** 1986. The hepatitis delta virus possesses a circular RNA. *Nature (London)* **323**:558–560.
20. **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 the delta antigen. *J. Virol.* **63**:1945–1950.
21. **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.
22. **Kuo, Y.-P., L. Goldberg, L. Coates, W. Mason, J. Gerin, and J. Taylor.** 1988. Molecular cloning of hepatitis delta virus RNA from an infected woodchuck liver: sequence, structure, and applications. *J. Virol.* **62**:1855–1861.
23. **Lazinski, D. W., and J. M. Taylor.** 1994. Recent developments in hepatitis delta virus research. *Adv. in Vir. Res.* **43**:187–231.
24. **Macnaughton, T. B., E. J. Gowans, S. P. McNamara, and C. J. Burrell.** 1991. Hepatitis delta antigen is necessary for access of hepatitis delta virus RNA to the cell transcriptional machinery but is not part of the transcriptional complex. *Virology* **184**:387–390.
25. **Nakabayashi, H., K. Taketa, K. Miyano, T. Yamane, and J. Sato.** 1982. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. *Cancer Res.* **42**:3858–3863.
26. **Owens, R. A., and T. O. Diener.** 1982. RNA intermediates in potato spindle tuber viroid replication. *Proc. Natl. Acad. Sci. USA* **79**:113–117.
27. **Rackwitz, H.-R., W. Rohde, and H. L. Sänger.** 1981. DNA-dependent RNA polymerase II of plant origin transcribes viroid RNA into full-length copies. *Nature (London)* **291**:297–301.
28. **Ryu, W.-S., H. J. Netter, M. Bayer, and J. Taylor.** 1993. Ribonucleoprotein complexes of hepatitis delta virus. *J. Virol.* **67**:3281–3287.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Schindler, I.-M., and H.-P. Mühlbach.** 1992. Involvement of nuclear DNA-dependent RNA polymerases in potato spindle tuber viroid replication: a reevaluation. *Plant Sci.* **84**:221–229.
31. **Sharmeen, L., M. Y. Kuo, and J. Taylor.** 1989. Self-ligating RNA sequences on the antigenome of human hepatitis delta virus. *J. Virol.* **63**:1428–1430.
32. **Sharmeen, L., M. Y.-P. Kuo, G. Dinter-Gottlieb, and J. Taylor.** 1988. The antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. *J. Virol.* **62**:2674–2679.
33. **Spiesmacher, E., H.-P. Mühlbach, M. Tabler, and H. L. Sänger.** 1985. Synthesis of (+) and (–) RNA molecules of potato spindle tuber viroid (PSTV) in isolated nuclei and its impairment by transcription inhibitors. *Biosci. Rep.* **5**:251–265.
34. **Suzuki, M.** 1990. The heptad repeat in the largest subunit of RNA polymerase II binds by intercalating into DNA. *Nature (London)* **344**:562–565.
35. **Symons, R. H., J. Haseloff, J. E. Visvader, P. Keese, P. J. Murphy, D. S. Gill, K. H. J. Gordon, and G. Bruening.** 1985. On the mechanism of replication of viroids, virusoids, and satellite RNAs, p. 253–263. *In* K. Maramorosch and J. J. McKelvey, Jr. (ed.), *Subviral pathogens of plants and animals: viroids and prions*. Academic Press, Orlando, Fla.
36. **Tabler, M., and M. Tsagris.** 1990. Viroid replication mechanisms, p. 185–205. *In* R. S. S. Fraser (ed.), *Recognition and response in plant-virus interactions*. Springer-Verlag, Berlin.
37. **Thompson, N. E., T. H. Steinberg, D. B. Aronson, and R. R. Burgess.** 1989. Inhibition of *in vivo* and *in vitro* transcription by monoclonal antibodies prepared against wheat germ RNA polymerase II that react with the heptad repeat of eukaryotic RNA polymerase II. *J. Biol. Chem.* **264**:11511–11520.
38. **Tsagris, M., M. Tabler, and H. L. Sänger.** 1991. Ribonuclease T1 generates circular RNA molecules from viroid-specific RNA transcripts by cleavage and intramolecular ligation. *Nucleic Acids Res.* **19**:1605–1612.
39. **Wang, K.-S., Q.-L. Choo, A. J. Weiner, J.-H. Ou, 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 delta virus genome. *Nature (London)* **323**:508–513.
40. **Zawel, L., and D. Reinberg.** Advances in RNA polymerase II transcription. 1992. *Curr. Opin. Cell Biol.* **4**:488–495.