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Hepatitis Delta Virus Genome Replication: a Polyadenylated mRNA for Delta Antigen

SEN-YUNG HSIEH, MEI CHAO, LAURA COATES, AND JOHN TAYLOR*

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

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Hepatitis delta virus (HDV) replicates its genome in the nucleus of an infected cell. However, an unsolved problem has been the identification in the cytoplasm of a putative mRNA for the synthesis of the only virus-coded protein, the delta antigen. We now report the characterization of an 800-base RNA that is cytoplasmic, polyadenylated, and antigenomic and that should direct the translation of the delta antigen. This RNA was about 500 times less abundant than full-length genomic RNA. We mapped the predominant 5′ terminus and also the 3′ site at which the poly(A) is added. At a point 15 to 20 bases upstream of the poly(A) addition site is the sequence AAUAAA, which could have been used as a signal for the polyadenylation. When an infectious cDNA clone of the whole HDV genome was changed at this site to UUUAAA, the clone was no longer infectious and it was unable to direct the synthesis of the delta antigen. These findings provided additional evidence that the polyadenylated RNA was at least the predominant method for the expression of the delta antigen. Apparently the HDV RNA was processed as if it were a host mRNA polymerase II transcript, although this did not necessarily indicate that HDV RNA was transcribed with this enzyme.

Hepatitis delta virus (HDV), in its genome structure and replication, is very much like the viroids and virusoids of plants (J. M. Taylor, Curr. Top. Microbiol. Immunol., in press). However, there are also some major distinctions: the genome of HDV is several times larger than that of the plant agents and codes, on its antigenomic strand (22, 35, 36), for a 22-kilodalton protein, the delta antigen, that is synthesized in infected cells (33). The protein is found both inside virions (8) and in the nucleus of the infected cell (28, 33), and it has been shown to be essential for genome replication (21); however, how it actually assists the viral replication is not yet known.

The major antigenomic RNA present in infected cells is a circular molecule (10, 18, 22, 25, 35). There is evidence to suggest that such a conformation would be unable to bind ribosomes (19). For the cell to translate a true delta-specific mRNA, we would expect an RNA that is (i) cytoplasmic, (ii) antigenomic and spanning the open reading frame, (iii) polyadenylated, and (iv) 5′ capped. At the same time, these expectations have to be consistent with the observations that in an infected cell, the majority of the HDV RNAs are present in the nucleus (33).

Previously, we reported the use of Northern (RNA) blot analysis to detect, in the liver of a single experimentally infected chimpanzee, relatively small amounts of an 800-base poly(A)-containing antigenomic RNA (10). We estimated that there were about 600 copies of this RNA per average liver cell, relative to about 300,000 copies of HDV genomic RNA. In other words, the amount of the putative mRNA was 500 times less than the amount of unit length genomic RNA. We were unable to detect this RNA by Northern blot analysis in several other samples tested. However, as reported here, we have now used several approaches to obviate this problem and have not only established the detection but have also characterized the RNA in more detail and proved by site-directed mutagenesis that its existence is essential for both delta antigen synthesis and for genome replication.

* Corresponding author.

MATERIALS AND METHODS

Plasmids and transfections. HDV sequences cloned into the RNA expression vector pGem4B (Promega Biotec) were synthesized by standard procedures (29). Cloning into the eucaryotic expression vector pSVL (Pharmacia) was as previously described (21). Briefly, the clones were as follows: pSVL(D3) contains a trimer of the HDV genome; pSVL(D2M) contains a dimer mutated at the unique *Eco*RI site within the coding region for the delta antigen; and pSVL(Ag) contains the region, using the nomenclature of Kuo et al. (22), from 224 (*Bgl*II) to 781 (*Xba*I), so as to express the delta antigen. We have also constructed the vector pCMV(D3), using a trimer of HDV inserted into the vector pBC12/CMV/IL-2 (12), as obtained from B. Cullen; an 870-bp fragment (from the unique *Hin*dIII to *Bam*HI sites) was removed from the vector prior to the insertion of a head-to-tail trimer of unit length HDV DNA, permuted relative to the unique *Eco*RI site.

Oligonucleotides. These were chemically synthesized. The HDV-specific oligonucleotides are listed in Table 1.

RNA extraction. RNA was extracted from the liver of an infected chimpanzee (10) by using a guanidine isothiocyanate procedure (11). For cultured cells, total RNA was extracted by treatment with pronase in the presence of sodium dodecyl sulfate (21). After one extraction with phenol and two with ether, the samples were precipitated with ethanol in the presence of salt and carrier dextran (Sigma Chemical Co.) (37). Some samples were additionally treated with DNase I (Promega) and repurified. In certain experiments, the cultured cells were first fractionated via Nonidet P-40 treatment to obtain nuclei and cytoplasm (13). The cytoplasmic RNA, after isolation, was further fractionated by using oligo(dT)-cellulose (Sigma) to separate out the poly(A)-containing RNA (13, 29).

Immunoblot analysis. Total cellular samples from COS7 cells at 5 days after transfection were denatured and then separated on gels of 15% polyacrylamide. The proteins were then electrotransferred to a PVDF membrane (Millipore) and analyzed, as previously described (21), to detect the delta antigen.

TABLE 1. HDV-specific oligonucleotides

Code	Position[a]	Sequence[b]
C	1152–1115	TGGGGGGGTG TAACTTCGAA GGTTGGATCG AGGGAG
D	1004–987	CTCTTCCCAG CCGATCCC
E	978–961	TCCCCAGAGT TGTCGACC
F	967–944	CGTCGACCCC AGTGTTTAAA GCGGG
G	1392–1426	GGGAGCCCCC TCTCCATCCT TATCCTTCTT TCCGA
H	1531–1549	CCGGCCACCC ACTGCTCGA

[a] Using HDV sequence of Kuo et al. (22).
[b] Underlined nucleotides represent deviations from Kuo et al.

Primer extension. Oligonucleotide H (see Table 1) was end labeled by using T4 kinase (Bethesda Research Laboratories, Inc.) and [γ-^{32}P]ATP (6,000 Ci/mmol; DuPont, NEN Research Products) and hybridized to about 1 μg of poly(A)-containing RNA, prior to extension in the presence of 50 μg of dactinomycin (Sigma) per ml, using as reverse transcriptase a molecular clone of that part of the enzyme of murine leukemia virus that lacks ribonuclease H (Bethesda Research), by standard procedures (29). The products were examined on a DNA sequencing gel relative to a homologous dideoxy-sequencing ladder (30) initiated with the same end-labeled primer.

PCR. In those experiments initiated with RNA, the reverse transcription was carried out under standard conditions (29) by using the enzyme from avian myeloblastosis virus (Life Sciences, Inc.). Portions of this product were then used as a template for the polymerase chain reaction (PCR) (16) by using the *Taq* polymerase (The Perkin-Elmer Corp.), as described previously (24). The reaction was carried out in 50 μl by using 30 to 40 cycles with a thermal cycler (Techne) set for 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. The primer combinations are described in the text and figure legends. In order to sequence the PCR products, we made use of asymmetric PCR (15) followed by dideoxy sequencing (30) with the *Taq* polymerase. For site-directed mutagenesis of the poly(A) signal, we again used PCR, this time with oligonucleotide F, which spanned the site and replaced the signal with UUUAAA, by using a modification of the method of Higuchi (16). The mutation in the resultant DNA was confirmed by nucleotide sequencing.

RESULTS

Northern analysis of HDV RNAs. Previously we reported a Northern analysis of the HDV-related RNA species in the liver of an infected chimpanzee (10, 32). Such data indicated the presence among the antigenomic RNAs of a minor species of about 800 bases. The results of a further characterization of this RNA are presented in Fig. 1A. By using chromatography on oligo(dT)-cellulose, we found that the 800-base species, unlike any other HDV-related RNA, was a poly(A)-containing species. The amount of this species was relatively low; we estimated 600 copies per cell relative to about 300,000 of the genomic unit length RNA, that is, 500 times lower. The strand-specific probes used in Fig. 1A showed that the RNA was antigenomic. Evidence that the species approximately overlapped the predicted coding region for the delta antigen was obtained by its inability to hybridize with a probe to the external sequence spanning the region from 655 to 962 (22) (data not shown).

We examined other RNA samples from infected chimpanzees and woodchucks (10) by the same Northern procedure but were unable to detect the same RNA species. Likewise, we were unable to detect this 800-base RNA in cultured woodchuck hepatocytes infected in vitro (33) or in cells transfected in vitro with a cDNA clone of the HDV genome (21). However, in certain experiments, at 4 days after transfection with an infectious cDNA clone (21) of mouse NIH 3T3 fibroblasts and a human hepatoma cell line, HuH7, we not only detected a similar species but were able to show by cell fractionation, using the detergent Nonidet P-40 (13), that this species was predominantly localized to the cytoplasm. The results for transfected NIH 3T3 cells are shown in Fig. 1B. Quantitation of the amount of the poly(A)-containing 800-base species in the cytoplasm indicated that it was 6,000 times lower than the amount of genomic RNA in the nucleus. This low amount has led us to conclude that our inability to detect the mRNA by this approach for certain other samples is not technical but represents an intrinsic feature of the biology of HDV replication (see below and Discussion).

In subsequent assays with a more sensitive detection procedure, the poly(A)-containing species was reproducibly detected in the RNA from cells infected or transfected with HDV.

Anchored PCR analysis of polyadenylated antigenomic HDV RNAs. Frohman et al. (14) have described a procedure which makes use of the PCR to amplify RNA species that are both related to a specific probe and poly(A) containing. They have called this procedure rapid amplification of cDNA ends or RACE. Others have referred to it as anchored PCR (23). As summarized in Fig. 2A, we applied this strategy to examine RNAs isolated from cells infected with HDV. Briefly, the poly(A)-containing RNAs were reverse transcribed by using primer A, the dT adaptor of Frohman et al. (14). Then the HDV-specific DNA products were selectively amplified by using primer B, the adaptor of Frohman et al., and the HDV-specific oligonucleotide, primer C. Such products were examined by slot analysis by using a second HDV-specific sequence, end-labeled oligonucleotide E. As shown in Fig. 2B, we obtained a positive result with an RNA sample from infected cells, lane 2, or transfected cells, lanes 5 and 6, in which the HDV genome was replicating. However, it was not present in either uninfected cells, lane 3, or in cells transfected with HDV sequences that were not replication competent, lane 4 (21). As a positive control, we used cells that had been transfected with a subgenomic clone of HDV, pSVL(Ag), that was designed to express a poly(A)-containing RNA species encoding the delta antigen, lane 1.

In order to confirm that the amplified DNAs were in fact derived from an HDV poly(A)-containing RNA, we took the product as used in lane 2 of Fig. 2B and submitted it to additional PCR amplification, this time with primers B and D, followed by direct dideoxy sequencing relative to end-labeled oligonucleotide D. The sequence obtained, as shown in the left panels of Fig. 2C, confirmed that the majority of the product was derived from RNA that was both antige-

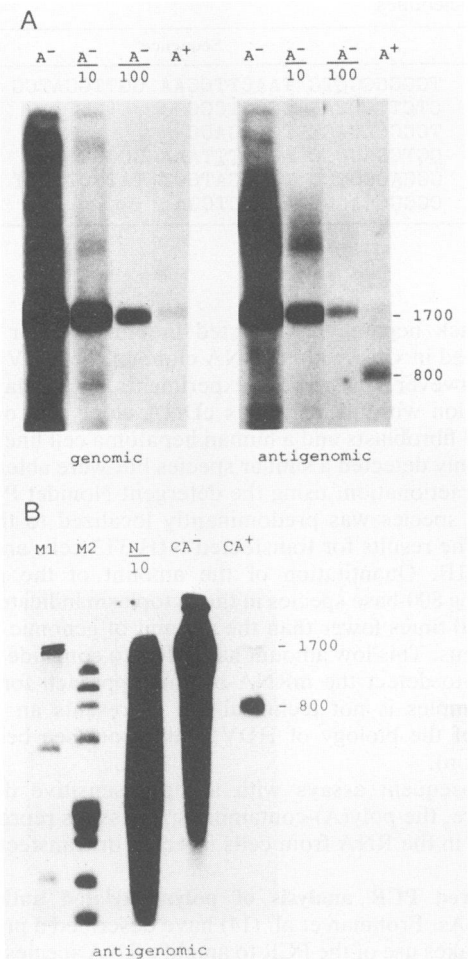

FIG. 1. Northern analysis of poly(A)-containing HDV RNAs. (A) RNA from the liver of a chimpanzee infected with both hepatitis B virus and HDV (10) was fractionated by chromatography on oligo(dT)-cellulose prior to glyoxalation and electrophoresis into a gel of 1.5% agarose. After electrophoretic transfer to a nylon membrane, hybridization was first carried out with a probe specific for the antigenomic RNA. After autoradiography (right panel), the filter was stripped and hydridized with a probe specific for genomic RNA, followed by autoradiography (left panel). The relative amounts of poly(A)-deficient, A^-, and poly(A)-containing, A^+, RNAs are indicated at the top. The lengths deduced for the major bands are indicated at the right side. (B) This shows a similar gel analysis but with the following differences. The RNA was from mouse 3T3 cells that had been transfected 4 days earlier with clone pCMV(D3). The cells were fractionated into nuclei and cytoplasm (13) prior to extraction of the RNA and the further separation of the cytoplasmic RNA with oligo(dT)-cellulose to yield a poly(A)-containing fraction, CA^+, and a poly(A)-deficient fraction, CA^-. Only a 10% amount of nuclear RNA, N/10, was analyzed. The gel was of 2.5% agarose, and the size markers were both *Hin*dIII fragments of phage lambda RNA, M1, and *Hae*III fragments of phage φX174 DNA, M2.

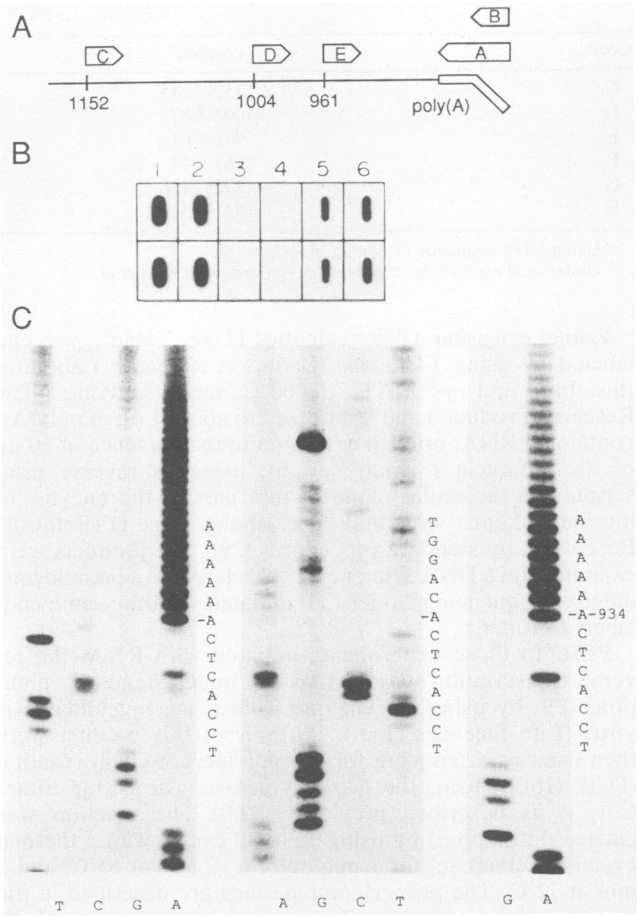

FIG. 2. Analysis by PCR to detect and characterize the poly(A)-containing antigenomic HDV RNAs. (A) Summary, in relationship to a poly(A)-containing antigenomic RNA, of the HDV-specific primers used (see Table 1 for more details). Primers A and B are the dT-adaptor and adaptor primers of Frohman et al. (14), respectively. (B) Slot analysis of RNAs from COS7 cells transfected with the following DNAs: 1, pSVL(Ag); 4, pSVL(D2M); 5, pSVL(D2M) and pSVL(Ag); and 6, pSVL(D3). RNAs from infected and uninfected primary woodchuck hepatocytes were used for lanes 2 and 3, respectively. The RNAs were reverse transcribed in the presence of primer A and then amplified by PCR by using primers B and C. The products were applied in duplicate to a nylon membrane and then hybridized by using as probe the end-labeled oligonucleotide E, followed by autoradiography. (C) Products used in lanes 2 and 1 further amplified by using primers D and B, followed by direct dideoxy sequencing, using as primer end-labeled oligonucleotide D, with results as shown at the left and right, respectively. At the center is shown the control, corresponding to the antigenome sequence. Deduced nucleotide sequences are shown adjacent to the sequencing ladders.

nomic HDV and poly(A) containing. It also identified the predominant site at which the poly(A) sequence began, as summarized in Fig. 3. This result, obtained with RNA from infected hepatocytes, was also obtained with the RNA from COS7 cells transfected with either pSVL(D3) (data not shown) or pSVL(Ag) (Fig. 2C, right panels). In the latter case, the whole HDV genome was not present and the RNA transcripts were DNA directed by using the vector promoter and, presumably, RNA polymerase II (pol II). In other words, the polyadenylation specificity was identical for this RNA and for the putative HDV mRNA generated during genome replication.

Figure 3 shows not only this adenylation site on the antigenomic RNA but also several features that support the hypothesis that this is part of a mRNA for the delta antigen. (i) The adenylation site is 76 bases downstream of the termination codon predicted for the 195-amino acid form of the delta antigen. (ii) The adenylation site is 15 bases

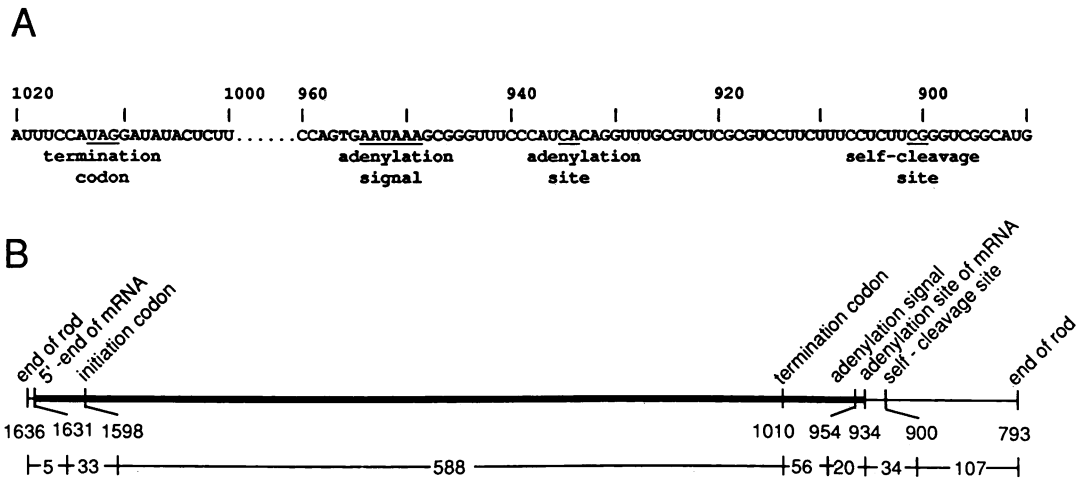

FIG. 3. Features of the antigenomic RNA of HDV. (A) Sequences around the adenylation site, as deduced from Fig. 2C, by using the HDV sequence information and numbering of Kuo et al. (22). HDV genomic and antigenomic RNAs have the ability to fold by base pairing into an unbranched rod structure (22, 35). (B) Precisely one side of the predicted unbranched rod structure of the antigenomic RNA, from position 1636 to 793. The diagram is drawn to scale, with the nucleotide positions and lengths deduced shown below in order to indicate the relationship of the features on the sequence. The deduced extent of the mRNA is indicated by the thicker line.

downstream of a sequence, AAUAAA, that has been found to be the consensus signal for polyadenylation of mRNA species (7). This sequence exists at no other location on either the genomic or antigenomic RNAs of HDV. Its location relative to the observed adenylation site is exactly within the consensus for the distance between a polyadenylation signal and the site of adenylation (7). It has also been found that such adenylation sites are frequently at the sequence CA (7); the site observed here is also CA, and there are, on either side, two more CA sites, apparently unused or less used. It should be noted, as indicated in Fig. 3, that the previously described site of in vitro self cleavage (31) is 34 bases downstream of the adenylation site.

In summary, our results show that signals exist on antigenomic HDV RNA that can direct polyadenylation, both of RNAs generated by RNA-directed RNA synthesis during HDV genome replication and of RNAs transcribed via DNA-directed RNA synthesis from pSVL(Ag) by pol II.

Determination of the 5′ end of the putative mRNA. A primer extension procedure was applied to the poly(A)-containing RNA from the cytoplasm of mouse 3T3 cells at 4 days after transfection with a cDNA clone of HDV. As shown in Fig. 4, we obtained a single band, lane 2. This band was not found when we used RNA from untransfected cells, lane 1. The same band was also obtained with transfected human hepatoma cell line HuH7 (data not shown), rather than mouse 3T3 cells. By comparison with an adjacent dideoxy-sequencing ladder, we interpreted the 5′ end of RNA as that predicted by the size of this extension product. (An alternative possibility, invoking RNA splicing, was considered as unlikely.) Our interpretation, as shown in Fig. 3, corresponded to position 1631, five bases away from one of the predicted ends of the unbranched rod structure. (From our data, we could not exclude the possibility that the 5′ end might be one nucleotide away, at 1632.)

The 5′ end determined by primer extension was, in addition, 33 bases upstream of the predicted initiation codon for the delta antigen. In combination with the determination of the adenylation site, we deduce that the mRNA should be 697 bases long, excluding poly(A). This is consistent with the Northern analysis of Fig. 1, which indicated about 800 bases, including the poly(A).

Effect of site-directed mutagenesis of putative polyadenylation signal. In the above studies, we have presented the detection and partial characterization of an RNA that could be the mRNA for the delta antigen. In order to further test this hypothesis, we made use of site-directed mutagenesis. The putative poly(A) signal, AAUAAA, was changed to UUUAAA by a PCR-based strategy, as summarized in Fig. 5A. This sequence was reconstructed back into a eucaryotic

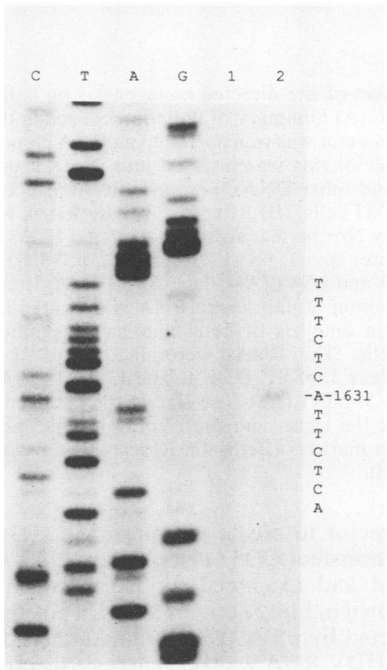

FIG. 4. Determination of the 5′ end of the polyadenylated antigenomic HDV RNA by primer extension. The RNA was obtained from mouse 3T3 cells transfected as for the sample in lane 6 of Fig. 2B, followed by oligo(dT)-cellulose chromatography. Lanes 1 and 2 represent primer extensions on poly(A)-containing RNAs, from mouse 3T3 cell before and after, respectively, transfection with HDV cDNA. CTAG indicates a dideoxy-sequencing ladder. All lanes make use of the same end-labeled oligonucleotide, H. At the right is the deduced sequence of genomic RNA.

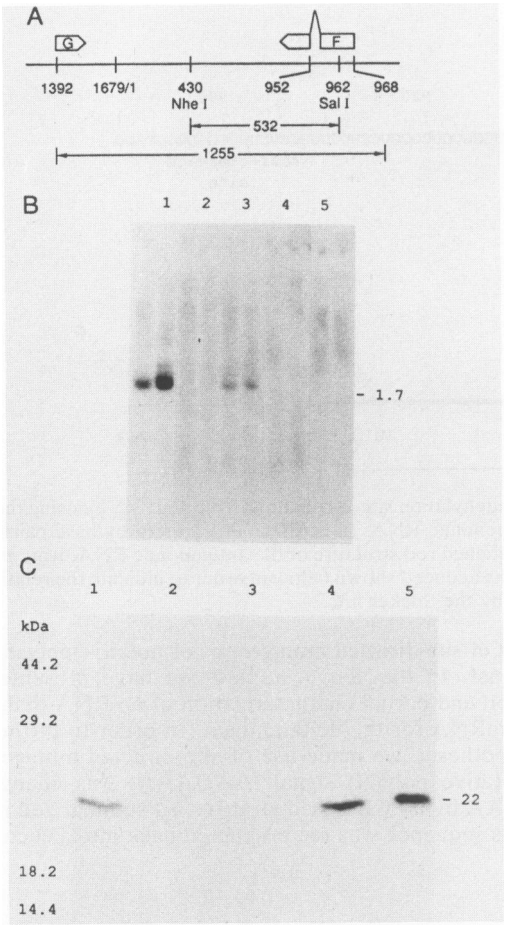

FIG. 5. Effect of site-directed mutagenesis on putative polyadenylation signal. (A) Summary of the primers used in the PCR-based strategy. The mutant was transferred to a cDNA clone of full-length HDV. A trimer of this was inserted into a eucaryotic expression vector. This and other DNAs were used to transfect, in duplicate, cultures of COS7 cells. (B) After 12 days the RNAs were extracted and assayed by Northern analysis for genomic HDV RNA species. The DNAs were: lane 1, pSVL(D3); lane 2, pSVL(D3 A^-); lane 3, pSVL(D3 A^-) and pSVL(Ag); lane 4, pSVL(Ag); and lane 5, no DNA. The position of unit length RNA is indicated at the left. (C) An immunoblot analysis of delta antigen as synthesized by the transfected cells. The DNAs were: lane 1, pSVL(D3); lane 2, pSVL(D2M); lane 3, pSVL(D3A^-); lane 4, pSVL(D3A^-) and pSVL (Ag); and lane 5, pSVL(Ag). The size of the detected delta antigen is indicated at the right, and the electrophoretic mobility of prestained protein markers (Bethesda Research Laboratories) is indicated at the left.

expression vector to create plasmid pSVL(D3 A^-), which was used to transfect COS7 cells. After 12 days, the RNAs were isolated and examined by Northern analysis, with results as shown in Fig. 5. Lane 1 shows a positive control of cells transfected by pSVL(D3), a nonmutated plasmid. The 1.7-kilobase HDV RNA was present, as expected from cells replicating the HDV genome (21). Lane 2 shows that the mutant did not allow such genome replication. Lane 3 shows the result of cotransfecting the mutant along with pSVL(Ag), a plasmid that contained only the ability to express the delta antigen (21). Clearly, this rescued the replication of the mutated genome, indicating that the mutation in the poly(A) signal had but a single consequence, one that could be relieved simply by the provision, in *trans*, of the delta antigen. Lanes 4 and 5 show cells transfected either with the antigen-producing vector, pSVL(Ag), or with no DNA, respectively. Separate immunoblot analyses of the transfected cells demonstrated that the mutant was unable to direct the synthesis of delta antigen (Fig. 5C, lane 3). Our interpretation of these studies was that the putative poly(A) signal was strictly needed because it directed the expression of the mRNA for delta antigen.

In summary, our results imply that a relatively minor 800-base species of polyadenylated antigenomic RNA may be the only template for translation of the delta antigen in HDV-infected cells. The *cis*-acting features of HDV involved in the poly(A) addition were theoretically and experimentally the same as those used for the processing of those cellular mRNA precursors generated by DNA-directed RNA synthesis with pol II. Of course, it does not necessarily follow that the HDV RNA transcription was by the same polymerase.

DISCUSSION

We have detected and characterized an antigenomic HDV-related RNA species that is cytoplasmic and polyadenylated and could be the mRNA for the synthesis of the delta antigen. The distance on the HDV sequence between the mapped 5′ end and the 3′ adenylation site was 697 bases (see Fig. 3B), consistent with these sites being on the polyadenylated 800-base RNA detected by Northern analysis. By site-directed mutagenesis, we showed that the RNA was essential for delta antigen synthesis and genome replication. We believe this result is more convincing than our previously reported inability to translate in vitro the poly(A)-selected RNA from the liver of the infected chimpanzee (32). Our observation that the molecule has a unique 5′ terminus will be useful in subsequent studies, since it implies the existence of a specific promoter element used in RNA-directed RNA synthesis. At this time we do not know whether the RNA is capped.

It remains to be explained why the amount of this mRNA is so low relative to unit length genomic and antigenomic RNAs, especially when the protein product is readily detectable. We know that the protein accumulates in the nucleus (21, 28, 33) and may be relatively stable, and we speculate that the low level of mRNA may be a consequence of regulation at two or more levels, including synthesis and stability.

Consider first the level of RNA synthesis. HDV genome replication takes place in the nucleus (33). Since the RNA for translation is processed through polyadenylation and also transported to the cytoplasm, there has to be regulation of these steps in the nucleus. HDV may not actually satisfy the definition of a virus, but it is negative stranded (1, 35, 36). As with negative-strand RNA viruses, such as influenza (20) and vesicular stomatitis virus (2), there has to be a differential utilization or regulation of RNA transcription: some RNA transcripts can be used for translation and others must remain as templates for additional rounds of RNA genome replication. It may be relevant that the above-mentioned negative-strand viruses have been shown to regulate their transcription by a virus-coded protein other than the RNA polymerase. HDV has only one known protein, the delta antigen, and it may have to function in this role. It is possible that this regulation is coupled to RNA synthesis and occurs at the level of polyadenylation relative to the site of in vitro self-cleavage (31), especially since the relevant signals are in close proximity (Fig. 3). Experiments are needed to test this

hypothesis, but clearly one of the outstanding gaps in our knowledge is the nature of the polymerase(s) used in the HDV RNA-directed RNA synthesis. The finding here that the mRNA is apparently processed as if it were a host mRNA precursor does not necessarily implicate pol II as the enzyme responsible for HDV transcription.

A second level of regulation might be at the level of stability of the cytoplasmic mRNA. Others have demonstrated many different factors which can affect the stability of mRNA species (6). However, we are attracted by one particular mechanism, described in a recent report of Kimelman and Kirschner (17). They observed the specific and efficient destabilization of a mRNA species by a combination of an excess of antisense RNA and a cellular activity that attacks double-stranded RNAs. This activity, known as RNA unwindase (4, 27), deaminates adenosine to inosine (3, 34), somehow leading to a rapid loss of polyadenylation and disappearance of the mRNA. It has already been implicated as a modifier of replicating negative-stranded RNA viruses (5, 9, 26) and maybe also of HDV RNAs (24). Also, it should be noted that when an HDV-infected cell undergoes breakdown of the nuclear membrane during mitosis, there will be the possibility of driving the HDV antigenomic cytoplasmic mRNA into a double-stranded hybrid with the much more abundant genomic RNA species. This, also, is a testable hypothesis.

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