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Review

Ribozyme activity in the genomic and antigenomic RNA strands of hepatitis delta virus

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Abstract. In the hepatitis delta virus, ribozymes are encoded in both the genomic strand RNA and its complement, the antigenomic strand. The two ribozymes are similar in sequence and structure, are most active in the presence of divalent cation and catalyze RNA cleavage reactions which generate a 5′-hydroxyl group and a 2′,3′-cyclic phosphate group. Recent progress has been made in understanding the catalytic mechanism. One key was a crystal structure of the genomic ribozyme that revealed a specific cytosine positioned to act as a general acid-base catalyst. The folding of the ribozyme in the context of the longer viral RNA is another area of interest. The biology requires that each ribozyme act only once, and mechanisms proposed for regulation of ribozyme activity sometimes invoke alternative RNA structures. Likewise, interference of ribozyme function by polyadenylation of the antigenomic RNA strand could be controlled through alternative structures, and a model for such control is proposed.

Key words. Catalytic RNA; RNA replicons; RNA processing; polyadenlyation; RNA secondary structure.

Introduction

In 1977, a new antigen was discovered in the livers of some patients with chronic hepatitis B virus infections [1]. Soon thereafter, it was found that this antigen appeared as the result of infection by a distinct virus, the hepatitis delta virus (HDV) [2]. Infection by HDV required either prior or concurrent hepatitis B virus infection; thus, HDV is a satellite virus of the hepatitis B virus [3, 4].

Studies of the virus revealed unusual features of the genome relative to other animal viruses. The genome of HDV is a small circular RNA molecule of 1679 nucleotides [5] that can form an elongated, unbranched, rod-shaped structure under native conditions [5, 6]. The formation of a partial duplex is a result of the high degree of complementarity between the two halves of the RNA circle [5], making it possible for roughly 70% of the sequence to be involved in basepairing over the length of this molecule. Similarities of the genome structure and biology of HDV to that of some of the plant virus satellites and viroids were noted [5, 7]. These comparisons became more striking with the discovery of self-cleaving sequences (ribozymes) in both the antigenomic and genomic strands of HDV RNA [8–10]. However, the HDV genome encodes a protein, the hepatitis delta antigen (HDAg) and, thus, is larger than the genomes of the plant virus satellites, which do not encode proteins [11]. Two forms of the HDAg are made: a small form (sHDAg), which is thought to be essential for replication of the HDV RNA [12], and a large form (lHDAg), which suppresses replication [13, 14] and promotes packaging of HDV RNA into new virus particles [15]. The lHDAg arises later in infection as a result of RNA editing at the stop codon of the sHDAg, which extends the carboxy-terminal end of the protein by an extra 19 amino acids [16].

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However, no enzymatic activity has been directly associated with either form of HDAg, and viral RNA replication is dependent on a host-encoded replicase activity. HDV may therefore be viewed as an intermediate between very simple RNA replicons without protein coding capacity, as is the case with viroids and some of the small plant virus satellites, and larger viruses that encode some of the enzymes essential to replication in a host cell [17]. A double rolling circle model has been proposed for the replication of the HDV RNA [5, 7, 11]. This mechanism was first proposed for replication of viroids and some other classes of small pathogenic RNAs [18]. It is hypothesized that the circular HDV genomic RNA serves as the template in a rolling circle mechanism to produce linear antigenomic-sense RNA. Longer-than-unit-length antigenomic RNA is cleaved into unit-length pieces via the antigenomic ribozyme, and the unit-length linear products are ligated by an as of yet unknown mechanism to form HDV antigenomic RNA circles. These antigenomic RNA circles also serve as a template in a rolling circle mechanism, the product of which is cleaved, via the genomic ribozyme, and ligated to produce genomic-sense RNA circles, thereby completing the cycle. Recleavage of the circular RNA by the ribozyme may be prevented through disruption of the ribozyme structure by base pairing with the partially complementary sequence which templates for the ribozyme found in the complementary strand of the virus [19].

It has been proposed that replication of the HDV RNAs may be performed by the host cell RNA polymerase II. Evidence for this comes from the inhibition of HDV RNA replication both by α-amanitin, an inhibitor of RNA polymerase II [20, 21], and by a monoclonal antibody specific for RNA pol II [21]. Additionally, in vitro replication of a portion of the HDV antigenomic RNA by RNA pol II in HeLa cell nuclear extracts has been demonstrated [22]. This replication was also found to be sensitive to α-amanitin, except in a cell line expressing a version of pol II that is resistant to the effects of α-amanitin [22]. One possible explanation for the ability of RNA pol II to replicate these RNAs may lie in their structure. The genomic and antigenomic HDV RNA circles are believed to form largely unbranched, partial duplex structures in vivo [5, 6]. It is possible, then, that these RNAs mimic DNA duplex recognized by transcription factors or RNA pol II. A more recent study provides evidence that synthesis of the full-length (1.7 kb) HDV antigenomic RNA and the shorter (0.8 kb) antigenomic-sense messenger RNA (mRNA) may occur through different molecular mechanisms [23]. Specifically, production of the mRNA for the HDAg was inhibited by α-amanitin, whereas production of the full-length RNA was less sensitive to α-amanitin. These results indicate the possibility that cellular RNA pol II is responsible only for production of the smaller mRNA and that a different polymerase is responsible for synthesis of the full-length antigenome [23]. The idea that different polymerase complexes are involved in replication and mRNA synthesis is also supported by a more recent finding that the HDAg binds directly to host RNA pol II and stimulates elongation; in nuclear extracts, the HDAg displaces negative elongation factor (NELF) and also directly stimulates transcription elongation [24]. Defining the enzymology of the replication process remains one of the most interesting quests in the study of HDV biology.

Involvement of ribozyme cleavage activity in RNA replication

Self-cleaving ribozymes, hypothesized to be responsible for processing linear multimer replication products into linear monomers, are found in both the genomic and antigenomic HDV ribozyme sequences. In vitro self-cleavage at a specific site was first reported in the antigenomic RNA of HDV [8], and soon thereafter, a cleavage site was reported in the genomic RNA [9, 10]. The locations of the sites are related by the self-complementary nature of the RNA circles, and in fact it was on this basis that the second site was initially predicted and tested [9]. In both cases, cleavage required a divalent cation and generated ends with a 5′-hydroxyl group and a 2′,3′-cyclic phosphate group. It was also apparent from those first in vitro studies that the cleavage activity resided in only a small fragment of the 1.7-kb sequence. A minimal sequence required for robust activity of the genomic HDV ribozyme required one nucleotide 5′ to the cleavage site and 84 nucleotides 3′ to the cleavage site [25, 26]. The antigenomic HDV ribozyme was of similar size to the genomic ribozyme [26]. Their size and the nature of their cleavage products place them in the category of the small, self-cleaving ribozymes. Other members of this group are the hammerhead, hairpin and *Neurospora* VS ribozymes. All of these self-cleaving ribozymes utilize a catalytic mechanism that involves attack of the oxygen of the adjacent 2′-hydroxyl group of the nucleotide 5′ to the cleavage site on the phosphorus of the scissile phophodiester bond (fig. 1). As would be expected based on their proposed critical role in replication, the self-cleavage activities of the sequences that define these ribozymes have been found to be essential for HDV RNA replication inside cells [27, 28]. In vivo, the HDAg has been found to increase the extent of self-cleavage of a primary transcript, although the ribozyme was not dependent on the HDAg for activity [29].

Ligation of replication products to form circles

The catalyst responsible for ligation of the linear monomer RNA products into their circular forms has not

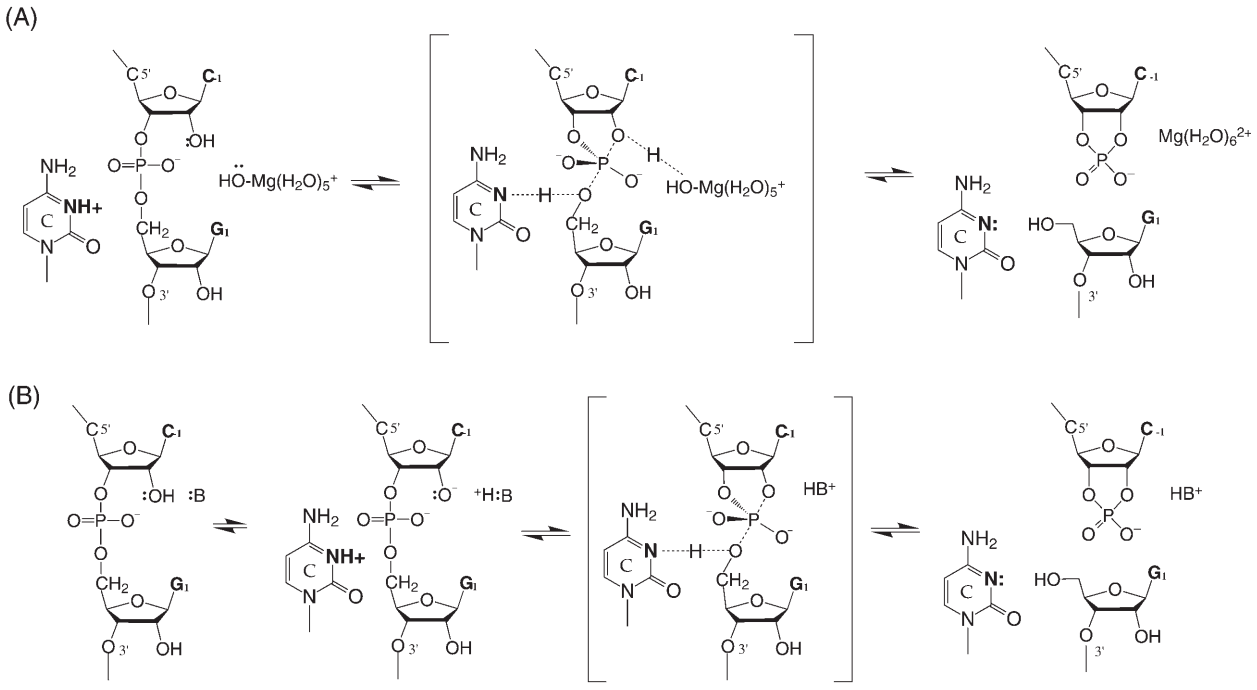

Figure 1. Chemical reaction and possible mechanisms for cleavage of the HDV ribozymes. The cleavage reaction generates a 5′-OH group and a 2′,3′-cyclic phosphate group. Details of the mechanism are under investigation; two related proposed mechanisms are shown. (*A*) Concerted deprotonation of the 2′-OH group and protonation of the 5′ leaving group occurs in the transition state. The conjugate base of a hydrated magnesium ion acts as the general-base catalyst, and the conjugate acid of the catalytic cytosine acts as the general-acid catalyst (general-acid general-base catalysis) [52]. (*B*) Deprotonation of the 2′-OH group occurs prior to the transition state, and the catalytic cytosine donates a proton to the leaving group oxygen (specific-base plus general-acid catalysis) [55]. The base (B:) has not been specified.

been identified. The possibility that the ribozymes catalyze the reverse reaction is attractive and reasonable. Although reports of self-ligation with HDV sequences have appeared in the literature, in vitro ligation of HDV RNAs via a mechanism involving solely the HDV ribozyme has yet to be conclusively demonstrated. A report of in vitro ligation of HDV RNA in which removing Mg^{2+} from the reaction was thought to shift the equilibrium towards ligation [30] was found to be an artifact induced by the quench conditions; however, it led to the discovery that the HDV ribozymes are stable and active in high concentrations of urea and formamide [25, 31]. A more recent report of self-ligation of an HDV ribozyme-containing transcript to form an RNA circle [32] was found to depend on running the polyacrylamide gels at low temperature. Anomalous migrating bands reported to be circular RNAs were instead heteroduplexes formed between linear RNA and circular DNA present in the reaction [33].
The inability of the HDV ribozymes to catalyze the ligation reaction probably reflects the absence of a binding site for the sequence 5′ to the cleavage site. Ligation of HDV ribozyme cleavage fragments does occur when the two ends of the RNA are brought into close proximity through base pairing of the ends with a partially complementary sequence from the viral RNA, but this only occurs in the presence of a base catalyst such as ethylenediamine [34, 35] or imidazole [I. H. Shih and M. D. Been, unpublished]. This reaction is unlikely to have relevance to ribozyme involvement in ligation since it necessitates disruption of the ribozyme structure; however, it supports the idea that a ligase may be capable of using the cleavage products directly as a substrate for ligation. Despite the inability to demonstrate ribozyme-catalyzed ligation in vitro, HDV RNA ligation in vivo has been shown [19, 36]. In vivo ligation of HDV RNA has been reported to be enhanced by the HDAg and requires the RNA binding activity of the HDAg [29]. A recent report demonstrated an activity in mammalian cells that was capable of ligating HDV ribozyme cleavage products in addition to ligating other RNAs with similar ends (5′-hydroxyl and 2′,3′-cyclic phosphate groups) [37].

Structure of the ribozymes

The secondary structures formed by the HDV genomic and antigenomic ribozymes are similar to one another and have been described as nested double-pseudoknot folds [38]. Both ribozymes have five duplex elements, called P1, P1.1, P2, P3 and P4 (fig. 2), with P3 and P1.1

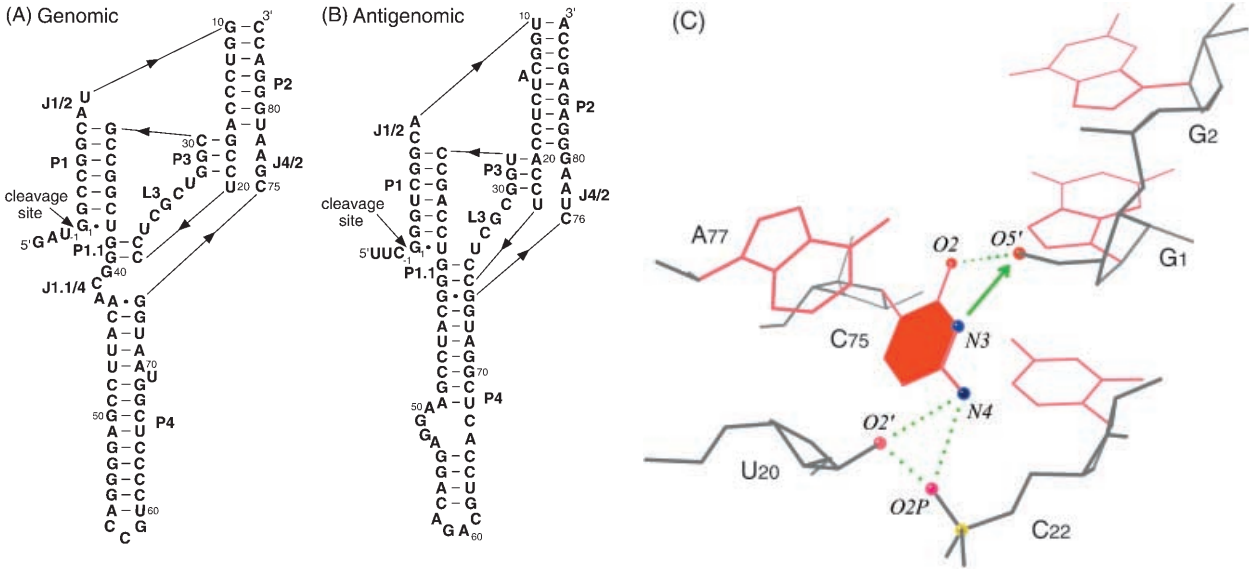

Figure 2. Sequence and secondary structure of the precursor forms of the genomic (*A*) and antigenomic (*B*) HDV ribozymes, along with a view of the active site from the crystal structure of a product form of the genomic ribozyme (*C*). (*A, B*) Nucleotides are numbered from the cleavage site. Self-cleavage sites and secondary structural elements are indicated. (*C*) Detailed view of interactions around C75 in the genomic ribozyme [48]. Dotted green lines indicate H-bond contacts with C75 in the product. It is proposed that in the cleavage reaction, the protonated form of C75 donates a proton to the 5′ oxygen leaving group (green arrow).

comprising a pseudoknot that falls within the pseudoknot defined by P1 and P2. Independently, P1.1 and P1 form a third pseudoknot with the cleavage site located at the stacked interface of these two duplexes. Support for P1, P2, P3 and P4 and their contribution to cleavage activity came from sequence comparison, mutagenesis and nuclease probing experiments [26, 39–41]. A crystal structure first revealed P1.1 [38], and mutagenesis has established that P1.1 is required for ribozyme cleavage activity in vitro [42, 43]. P2 can be shortened, but this results in a decrease in in vitro cleavage activity and a less stable ribozyme. P4 can be replaced with a short hairpin loop [40, 44] or with the RNA hairpin targeted by the U1A protein [38, 45], with little or no loss of activity. The sequences connecting these paired regions are represented as single stranded in the secondary structures, although many are involved in important non-Watson-Crick interactions. Active bimolecular forms of the ribozymes can be generated by separating the RNA at J1/2 or L4 [46, 47].

The structure of a crystal of a genomic HDV ribozyme 3′ cleavage product was solved by Ferré-D'Amaré et al. [38, 48]. The structure provides a picture of the HDV genomic ribozyme as a highly compact enzyme with an active site buried within the molecule. The duplex elements come together in two parallel coaxial helices formed by the stacking of P1, P1.1 and P4 as one helix and P2 and P3 as the other. The two helices are 'laced' together by five strand crossovers between the helical stacks [38]: three from J1/2, J4/2 and the connection between P1 and P3, and two more from the P1.1 helix, which takes the sequence at the end of P3 (previously assigned to L3) and tucks it between P1 and P4. The 5′ end of the RNA is buried between the two coaxial helices in a pocket formed by an arch of P1 and P3 on a floor of P1.1 and walled in by segments of J4/2 and L3. This pocket is thought to define the active site of the ribozyme. Because the structure is of the 3′ cleavage product, details of how the scissile phosphate and the nucleotide 5′ to the cleavage site are positioned within the active site await further structural studies.

Mechanism of the cleavage reaction

RNA cleavage by the HDV ribozymes results in a 5′-hydroxyl group and a 2′,3′-cyclic phosphate group, suggesting that the mechanism involves activation of the adjacent 2′-hydroxyl group for attack on the phosphorus at the cleavage site (fig. 1). Ribozymes, as with protein enzymes, are likely to use multiple catalytic strategies to promote this reaction.

The HDV ribozymes require divalent cation for optimal activity [8–10]. As with other ribozymes, both structural and catalytic roles for divalent cations are possible, and it is difficult to distinguish between these roles. The crystal structure does not reveal a strongly bound metal ion in the active site, suggesting that the product form of the ribozyme lacks a high affinity site for divalent cation. However, the electron density suggested the possibility of

a weak metal ion binding site. Because the crystalized sequence lacks both the scissile phosphate group and the nucleophilic 2′-OH group (both of which are potential metal ligands as well as sites for metal ion action), it is possible that a precursor form of the ribozyme may bind metal ion more tightly at the active site [38]. Thiophilic metal ion rescue experiments with a thiophosphate at the cleavage site were nondefinitive in this regard [49, 50]. Nevertheless, there is indirect biochemical evidence, for example a change in metal ion specificity with cleavage of a 2′,5′-phosphodiester linkage [51], that is consistent with a potential catalytic metal ion binding site located at or near the active site. A model of general-acid general-base catalysis has been proposed in which a hydrated Mg^{2+} acts as a general-base catalyst in the cleavage reaction [52]. It was found that the genomic ribozyme displayed a measurable level of cleavage activity in 1 mM EDTA and 1 M NaCl when Mg^{2+} was omitted from the reaction [52]; the reaction followed a rate-law definition of general-acid catalysis under these conditions, which was taken as evidence to support the involvement of a hydrated Mg^{2+} as a general-base catalyst in the normal reaction. Whereas alternative explanations for the inverted pH-rate profile under these conditions have been suggested [53], the discovery of divalent cation-independent cleavage activity in the genomic HDV ribozyme is significant and provides a new approach for investigating the contributions of divalent cations to the reaction. A relatively minor modification to the HDV antigenomic ribozyme sequence confers similar divalent cation-independent cleavage activity on this ribozyme in 1 M NaCl; therefore, this interesting variation of the normal reaction is a feature of both ribozymes [53].

A significant contribution of the HDV ribozymes to the understanding of RNA catalysis included identifying the involvement of RNA side chains in acid-base catalysis [38, 52, 54, 55]. In particular, cytosine 75 in the genomic ribozyme (C76 in the antigenomic ribozyme), which was known to be important for cleavage activity [56–58], was seen to be provocatively positioned in the active site of the genomic ribozyme 3′ product crystal structure [38] (fig. 2C). It was suggested that this cytosine might act as a base catalyst to extract the proton from the 2′-hydroxyl group of the nucleotide 5′ to the cleavage site [38, 59]. It was recently pointed out that the crystal structure is strongly suggestive of a mechanism in which the cytosine, acting as a general-acid catalyst, protonates the 5′ leaving-group oxygen [52]. The latter mechanism is attractive as it is consistent with the available structural data as well as with information indicating that the difficult step in this chemistry is the breaking of the 5′O–P bond rather than formation of the 2′O–P bond [60, 61].

Mutagenesis and exogenous base rescue experiments support the hypothesis that C75 in the genomic ribozyme and C76 in the antigenomic ribozyme participate in the rate-determining step of the reaction. A decrease in the apparent pK_a of the self-cleavage reaction was observed when C76 was changed to adenine in the antigenomic ribozyme [54] or with the equivalent C75A mutation in the genomic ribozyme [52]. This shift in the apparent pK_a correlated well with the difference in pK_a of cytosine (~4.2) versus adenine (~3.5), suggesting that ionization of the base affected the rate of the reaction. However, the overall rate of the reaction decreased much more than would be expected if the only effect of the mutation was to change the pK_a of an active site residue by 0.7. In an antigenomic HDV ribozyme with either a C76U or a C76G mutation, imidazole buffer or exogenous cytosine rescued cleavage activity [54]. The apparent pK_a of the reaction in imidazole buffer was the same as the pK_a of imidazole ($pK_a = 7$), suggesting that the rate was dependent upon the ionization state of imidazole. Rescue by exogenous cytosine was also examined using a C76Δ (deletion) mutant; in this case, rescue resulted in an apparent pK_a for the reaction consistent with the lower pK_a of cytosine versus imidazole (apparent $pK_a = 5.2$ at 50 mM cytosine) [55]. Together, these data were interpreted as evidence that imidazole in the rescue reaction mimics the function of C76 in the intact ribozyme and that the rate constant of the rate-determining step is dependent upon the ionization state of either the cytidine side chain in the wild-type sequence or the exogenous base in the rescue reaction.

Two lines of investigation support a mechanism in which imidazole or cytosine catalyzes proton transfer in the rate-determining step and thus acts as a general acid-base catalyst [55]. There is an apparent solvent isotope effect in D_2O, and proton inventory experiments suggest that a single proton is ‘in flight’ in the transition state. A Brønsted linear free-energy relationship using imidazole and three other imidazole-like bases (with pK_a's of 5.5, 6.6, 7.0 and 7.8) yielded a slope (β) of 0.5. β (the Brønsted value) is a measure of the change in effective charge in going from the ground state to the transition state. A β (or α, where $\alpha = 1 - \beta$) of 0.5 is consistent with the exogenous base acting either as a Brønsted base in a mechanism of general-base catalysis or as a Brønsted acid in a mechanism of specific-base plus general-acid catalysis. If these data stand up to further inquiry, the HDV ribozymes will have provided evidence that nucleobases in RNA can participate directly in general acid-base catalysis. The possibility that RNA catalyzes similar reactions in other ribozymes must also be considered.

Sequence variation in HDV

Natural sequence variation in the HDV genome is a potential source of information that can help shed light on structure-function relationships in the ribozyme. HDV clinical isolates have been divided into three genotypes

based on relatedness of their sequences [62]. Of these, genotype I is the most widespread, with isolates having been discovered in Europe, Asia, the Middle East, the South Pacific, North America and Africa [63, 64]. HDV genotype I has been associated with widely variable pathogenesis [62, 64]. Genotype II was first discovered in Japan, followed later by an isolate in northern Taiwan [65]. Sequence analysis of additional isolates in Taiwan and Okinawa caused the genotype II group to be further subdivided into genotypes IIa (composed of the first two isolates) and IIb (composed of the latter isolates) [66]. Genotype II is of fairly low prevalence even though the population in the regions from which the isolates have been obtained have a high incidence of hepatitis B infection (as high as 15–20% of the population can be carriers of HBV). Genotype II HDV seems to cause less severe hepatitis than genotype I, resulting in more favorable outcomes in patients with long-term bouts with the disease [65]. Genotype III is the lowest frequency group, with isolates having been found only in northern South America. This genotype, however, has been associated with severe and highly fatal hepatitis. Three isolates of genotype III have been sequenced and analyzed. From this analysis, it appears that genotype III is the most highly divergent of the three genotypes [62]. HDV sequences can vary widely between genotypes. In fact, even the ribozymes and HDAg coding regions from different genotypes can have fairly divergent sequences [65]. One study found that the HDAg from an HDV genotype III clone was unable to complement the HDAg activity of an HDV genotype I clone because the genotype III HDAg was unable to activate replication of genotype I RNA [67]. A study which compared a large number of HDAg sequences found that, with the exception of the isoprenylation site, the 19 amino acid carboxy-terminal extension that is unique to the lHDAg is very divergent between the three genotypes [64]. Whether or not this plays any role in factors such as severity or infectivity of different genotypes remains unclear [64].

Variation within the HDV ribozymes

The genomic and antigenomic ribozyme sequences are two of the most highly conserved regions of RNA sequence in HDV clinical isolates [62]. Sequence comparison of the ribozymes from clinical isolates supports the proposed secondary structure [68, 69]; using 22 full-length HDV clinical isolates deposited in the GenBank database, those comparisons are updated here (fig. 3) and interpreted with respect to the new structural data and catalytic mechanism. In the case of the genomic ribozyme sequences, one or more base differences were observed at 26 of the 86 nucleotide positions (fig. 3A). By far, the largest number of differences (17 of the 26) occurred in the P4 duplex and its associated L4 loop. Because this region has been found to be largely dispensable for cleavage activity in both HDV ribozymes [40, 41], it may not be surprising that most of the differences occur here; however, the sequence variation in this region supports the idea that the structure of P4, rather than its sequence, is critical to the biology of the virus even if it is not required for in vitro activity. Three differences are found in P2, two of which are responsible for a change from a U-A base pair to a C-G pair in one isolate (Peru-1; fig. 3A). The third difference in P2 creates a G·G mismatch at the very 3′ end of this duplex and, therefore, would not be expected to significantly decrease the stability of the duplex (M28267; fig. 3A). The L3 loop (or J1.1/2, nts 23–27) is surprisingly variable in the genomic ribozyme, although there seems to be a strict requirement for a length of five nucleotides (fig. 3A). In the crystal structure [48], this region is not well ordered, and the bases of nts 23, 26 and 27 are rotated away from the bulk of the structure, whereas those of 24 and 25 stack to the inside. It is possible that in the precursor RNA, portions of L3 might interact with sequence 5′ to the cleavage site [48]; in the genomic sequence, variation does occur at positions –2 and –3 relative to the cleavage site (fig. 4A), but no obvious pattern of covariation with sequence in L3 is evident to suggest specific contacts dependent on base-base interactions. A sequence difference that occurs in the J4/2 region (G76→A) of the Peru-1 isolate (fig. 3A) is consistent with mutagenesis data. A G76-to-U change had only a small effect (~15-fold) on self-cleavage activity of a genomic ribozyme [57]. Interestingly, in the crystal sructure this base is flipped out into solvent to make one of only two RNA-RNA crystal contacts [48]. In addition, it is susceptible to T1 nuclease cutting in solution, suggesting that it is also likely to be accessible to solvent under reaction conditions [39]. Extrusion of this base appears to be a key feature of this region of the structure and is associated with an inversion of the sugars of C75 and G76. This feature contributes to a high negative charge density in the vicinity of cytosine 75 that could be important for perturbing the pK_a of that base [48]. Of the 10 non-base-paired positions in L3 and J4/2, portions of which form the active site, 4 are invariant (fig. 3A) and three (C75, A77 and A78) line the active site pocket.

With the antigenomic ribozyme sequences, one or more differences were observed at 20 of the 90 nucleotide positions (fig. 3B). As was the case for the genomic ribozyme sequences, the largest number of differences (13 out of 20) occurred in the P4 duplex and its associated L4 loop. Of the remaining differences, four occur in P2. Two of these are in the Peru-1 isolate and are responsible for a change from a C-G base pair to a G-U pair (fig. 3B). The other two differences in P2 are more interesting and occur together in one isolate (Somalia; fig. 3B). Their effect appears to be elimination of the bulged adenosine that in-

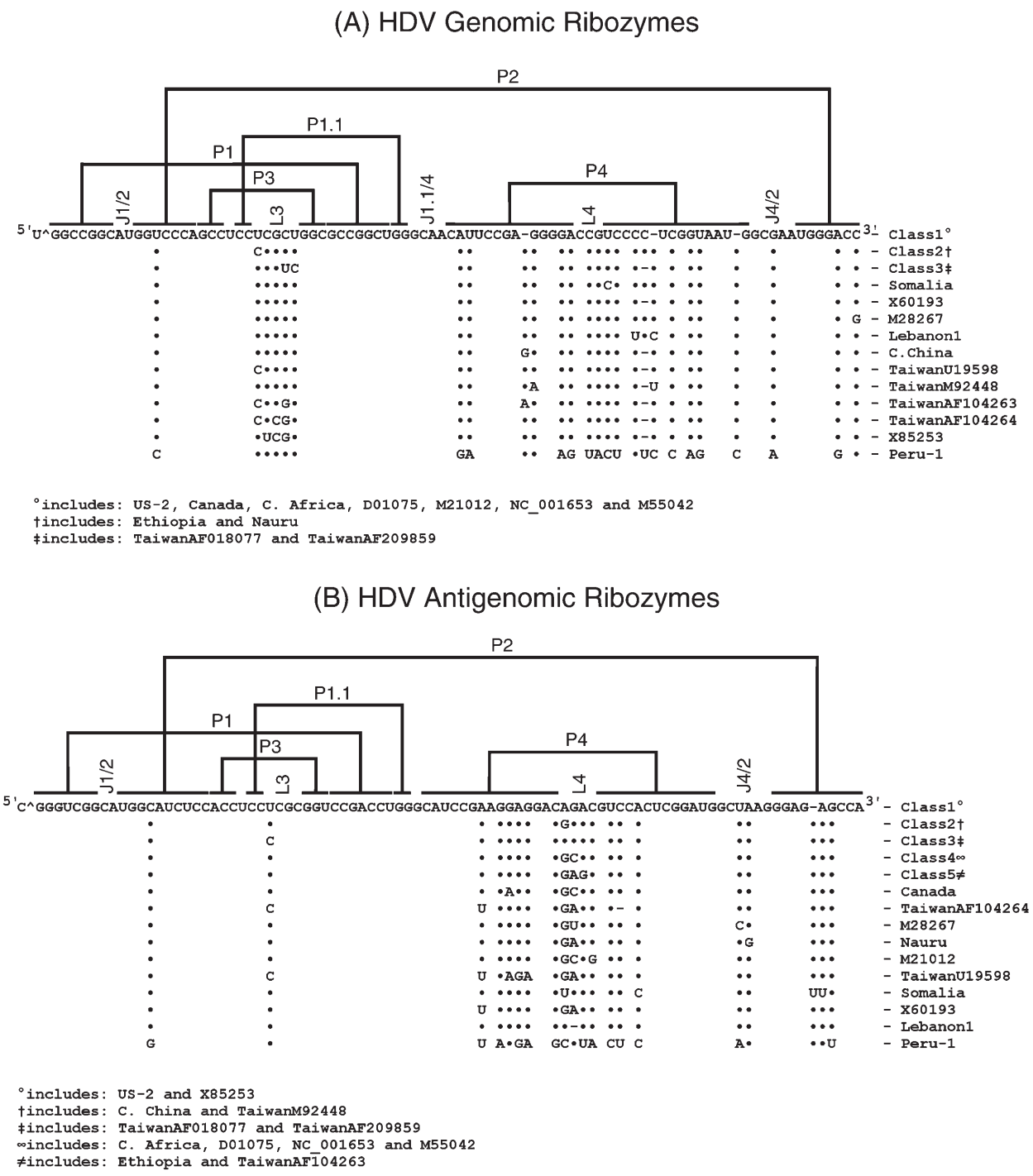

Figure 3. Sequence comparisons of HDV genomic (*A*) and antigenomic (*B*) ribozymes from clinical isolates. Sequences corresponding to ribozyme secondary structural elements are indicated with lines above the sequence. The self-cleavage site is marked (^). Only nucleotide positions where one or more changes occur are noted; at a position with variation, a dot (•) indicates identity with the reference sequence. Occasional gaps were introduced to facilitate alignment and are indicated by a dash (–). The name (or partial accession number) of each isolate is shown on the right. All sequences were obtained from the GenBank database.

terrupts P2 in the antigenomic ribozyme (fig. 2B) together with introduction of a U · U mismatch at nearly the same position. Previous in vitro mutagenesis data suggested that a bulged nucleotide in P2 facilitated folding of the antigenomic ribozyme [70]; thus, the sequence information suggests that a disruption in P2 may be important biologically. In contrast to the genomic ribozyme sequences, the sequence of L3 is nearly invariant in the antigenomic ribozyme and is consistently four nucleotides long (versus five in the genomic sequence) (fig.

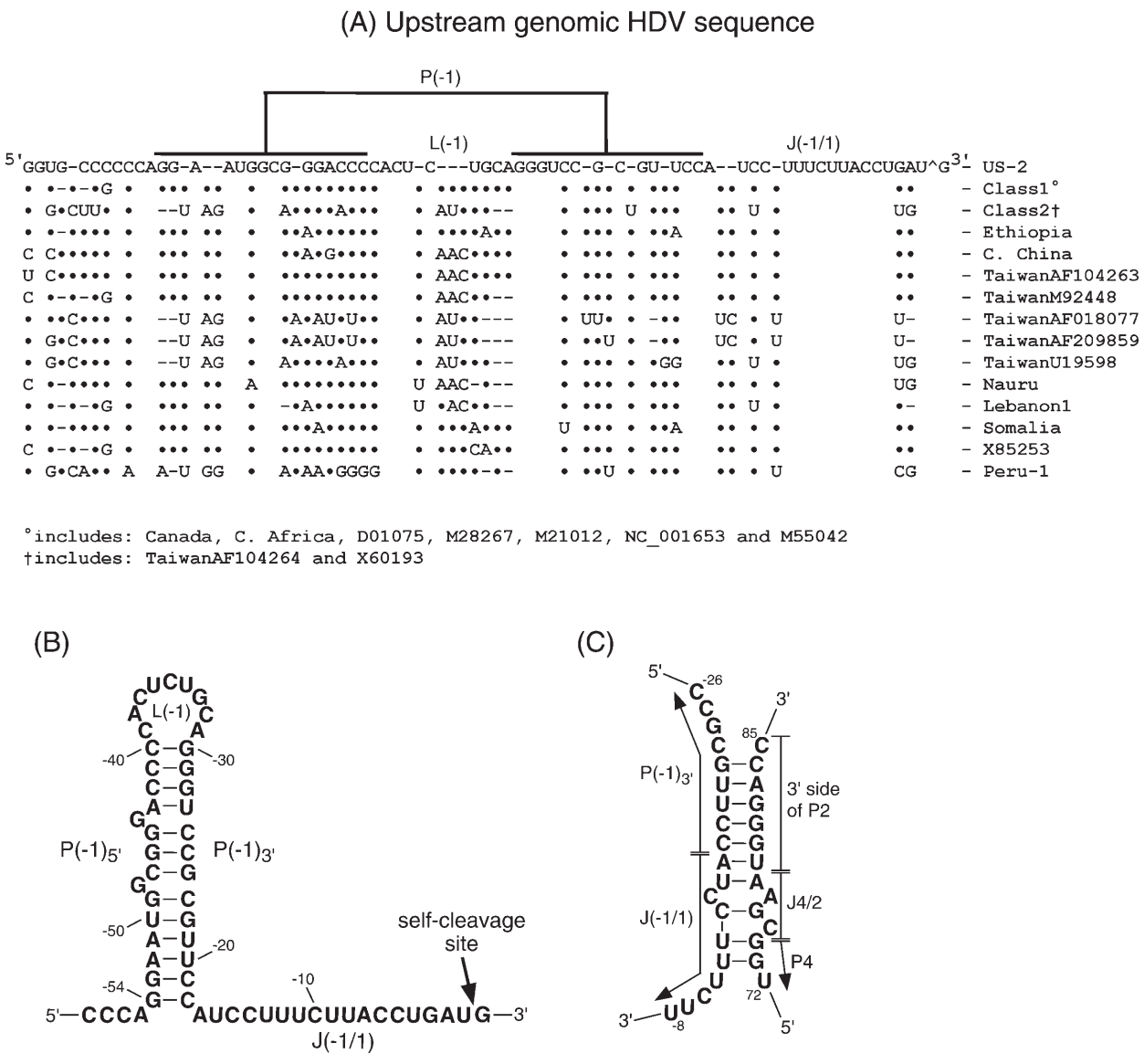

Figure 4. Sequence comparison and possible secondary structure upstream of the HDV genomic ribozyme self-cleavage site. (*A*) Comparison of clinical isolate sequences obtained from the GenBank database. Sequences corresponding to proposed secondary structural elements are indicated with lines above the sequence. See the legend to figure 3 for an explanation of symbols. The name (or partial accession number) of each isolate is shown on the right. (*B*) Sequence and proposed secondary structure of the P(–1) duplex [72]. (*C*) Alternative interaction between sequence upstream of the self-cleavage site and a portion of the genomic ribozyme. Ribozyme and upstream sequence secondary structural elements are labeled. All nucleotides are numbered from the cleavage site.

3B). Insertion of a U at the 3′ end of L3, making it more genomic-like, had little effect (1.2-fold decrease) on in vitro self-cleavage activity [71]. The one difference that is observed in the antigenomic L3 sequence, U26 to a C, occurs in three of the isolates (fig. 3B). The genomic ribozyme also has a C at this position with about the same frequency, suggesting a possible preference for a pyrimidine; nevertheless, a U26A mutation in the antigenomic ribozyme resulted in less than a twofold reduction in self-cleavage activity [58]. Two final sequence differences occur in the J4/2 region of three different isolates (fig. 3B). Variation at position U77 (to C and A) is consistent with in vitro studies of mutants with base changes at this position, where only small effects on activity were observed [58]. More interesting, however, is the one example of an A78 to a G difference (fig. 3B). In the genomic ribozyme, no changes are seen at this position (A77), and an A77U mutation resulted in a 10^3-fold reduction in the rate of cleavage [57]. In the antigenomic ribozyme, an A78C mutation reduced self-cleavage activity by approximately the same amount [58]. With the genomic ribozyme, where the extent of cleavage during transcription, but not the rate of the reaction, was followed for all three substitutions at this position, only the

A77G mutant cleaved to completion [56]. A small or moderate decrease in the rate of the reaction may not have been detected in those experiments, however. In the genomic ribozyme crystal structure, this adenine stacks on cytosine 75, its N-3 position participates in a ribose zipper interaction, and its exocyclic amino group faces the active site. The stacking and ribose zipper interactions would be expected to be sequence independent, and a cytosine amino group might be expected to substitute for the adenine amino group. Thus, it is not obvious why mutations at this position have such a large effect. Additional studies on A78G in the antigenomic ribozyme (A77G genomic) appear to be warranted. Both C76 and A79 (equivalent to C75 and A78 in the genomic ribozyme) are invariant (fig. 3B); as with A78, a possible role for A79 in catalysis has not been ruled out.

Sequence upstream of the cleavage site affects folding of the genomic ribozyme

Studies aimed at defining the sequence required for genomic ribozyme activity revealed inhibition of cleavage activity by flanking sequences [25]. Recently, a specific sequence upstream of the cleavage site was defined (nts –15 to –24) and shown to inhibit genomic ribozyme self-cleavage activity in vitro by forming an alternative pairing with the 3′ side of P2 [72] (fig. 4C). It is interesting to note that a nearly identical pairing between the 3′ side of P2 of the genomic ribozyme and exactly the same upstream sequence was a feature of a secondary structure first proposed for the genomic HDV self-cleaving sequence [10]. The same upstream sequence can also form a hairpin loop, termed P(–1) [72] (fig. 4B), with a sequence further upstream (nts –40 to –54) [72]. When P(–1) can form, the ribozyme is active; thus, the 3′ half of P(–1), designated $P(-1)_{3'}$, is inhibitory only when the 5′ half, or $P(-1)_{5'}$, is not present [72] (fig. 4B). It was proposed that the $P(-1)_{5'}$ sequence thus facilitates the formation of the catalytic fold of the genomic ribozyme [72].

It is not necessarily apparent what function the complementarity between $P(-1)_{3'}$ and the $P2_{3'}$ portion of the ribozyme might serve. One suggestion is that this alternative pairing, which disrupts the ribozyme, could provide a means to ensure that following ligation, the ribozyme remains inactive so that circular products are not relinearized [72]. Sequences upstream of the genomic ribozyme were compared for the available HDV clinical isolates [72], and this alternative pairing feature was found to be conserved. This suggests that the interaction might be biologically important. A similar sequence comparison is shown in figure 4A. As noted by Chadalavada et al. [72], there is high sequence conservation in the pyrimidine-rich stretch of nucleotides immediately 5′ to the genomic ribozyme cleavage site, J(–1/1). A number of changes are seen in the L(–1) sequence; however, there seems to be maintenance of the loop size to between 8 and 12 nts. The $P(-1)_{3'}$ sequence is more highy conserved than that of $P(-1)_{5'}$; however, of the changes that occur in either sequence, most are compatible with maintenance of the overall structure of the P(–1) duplex.

Sequence upstream of the antigenomic ribozyme

The self-complementary nature of the HDV genomic and antigenomic RNAs means that significant secondary structural features in one strand are likely to be mimicked in the other. In the antigenomic RNA, a P(–1)-like pairing located at the position equivalent to that seen in the genomic sequence is a feature of all of the clinical isolates (fig. 5A, B). Antigenomic-sense RNA, in addition to being processed by the ribozyme to form the template for genomic RNA synthesis, is cleaved and polyadenylated in the production of the mRNA for the delta antigen protein [4, 73]. The cleavage/polyadenylation site (CA) is located 32 nts upstream of the antigenomic ribozyme cleavage site [74] and falls within the hairpin loop of P(–1). Whereas the antigenomic P(–1) is approximately the same size as the genomic P(–1), it differs in that in all of the isolates examined, there is an internal bulged loop interrupting the pairing (fig. 5B). The 5′ side of this bulged loop consistently contains most of the AAUAAA polyadenylation signal for the antigenomic mRNA [74] (fig. 5A, B). In addition, the $P(-1)_{3'}$ sequence is nearly entirely composed of a previously identified G/U-rich sequence that has been found to be important for polyadenylation of the HDV mRNA [75] (fig. 5A, B).

As was the case for the genomic RNA, the 3′ side of P(–1) in the antigenomic RNA could potentially pair with P2 of the antigenomic ribozyme. A possible $P(-1)_{3'}$/P2 interaction for the US-2 isolate is depicted in figure 5C. There are alternative possible alignments for this pairing, most with more mismatches than are seen in the case of the genomic sequence. However, for the Peru-1 isolate, depending on how the $P(-1)_{3'}$/P2 pairing is drawn, there could be a run of at least 11 consecutive base pairs.

The sequences upstream of the cleavage site in the antigenomic RNA of clinical isolates were also compared (fig. 5A). As with the genomic RNA, the antigenomic J(–1/1) sequence immediately 5′ to the cleavage site is very highly conserved and, in this case, is composed solely of pyrimidines. The hairpin loop sequence, L(–1), is more highly conserved than in the genomic RNA, and always contains the CA polyadenylation site. Most variations that occur in $P(-1)_{3'}$ maintain the G/U-rich nature of this side of P(–1) (fig. 5A), and in addition, the potential for duplex structure in this region is retained despite most of the sequence differences in either side of P(–1). Bulging or unpairing of at least a major portion of the

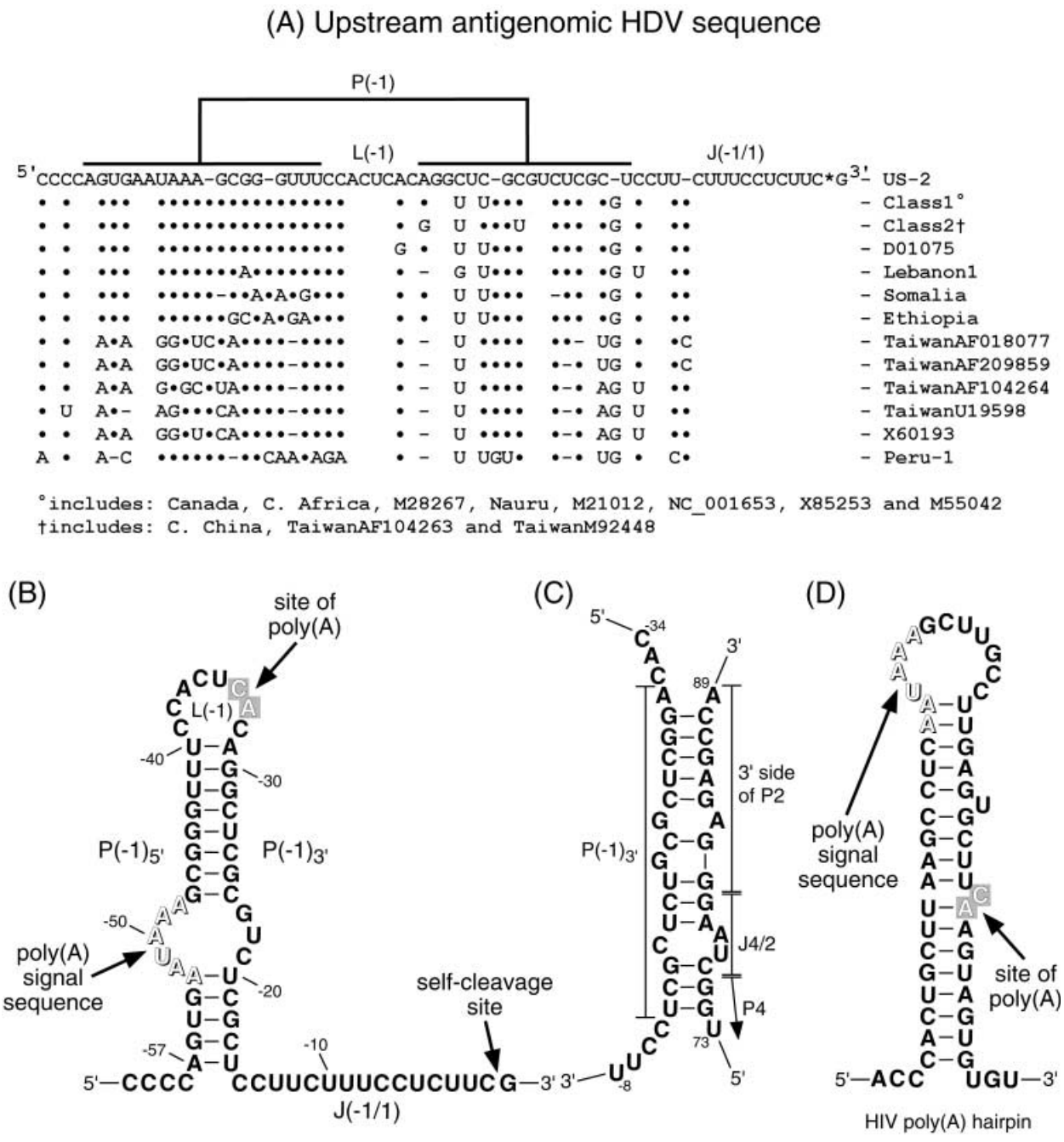

Figure 5. Sequences and possible secondary structure upstream of the HDV antigenomic ribozyme self-cleavage site, and comparison to the poly(A) hairpin of HIV. (*A*) Clinical isolate sequences obtained from the GenBank database. Sequences corresponding to proposed secondary structural elements are indicated with lines above the sequence. See the legend to figure 3 for an explanation of symbols. (*B*) Sequence and proposed secondary structure of the P(–1) duplex. The poly(A) signal sequence, site of poly(A) addition and self-cleavage site are indicated. (*C*) Proposed alternative interaction between sequence upstream of the self-cleavage site and a portion of the antigenomic ribozyme. Secondary structural elements are labeled. (*D*) Sequence and secondary structure of the HIV poly(A) hairpin. The poly(A) signal sequence and site of poly(A) addition are indicated.

AAUAAA sequence at the bottom of the hairpin is a feature of all of the sequences (fig. 5A, B). In the antigenomic sequence, the potential to form a P(–1) duplex could facilitate the folding of the ribozyme as proposed for the genomic ribozyme [72]. It is noticeable, however, that the potential to form this hairpin also positions key sequences required for polyadenylation within distinct secondary structural elements. This arrangement is intriguing because there is a need to regulate polyadenylation in HDV such that it occurs on the message and not on the replication product.

A model for regulating polyadenylation in HDV

There exist other examples where polyadenylation signals reside within regions of defined RNA secondary structure [76–79]. A well-studied example is that of the type I human immunodeficiency virus (HIV-1), where, similar to the situation proposed above for the hepatitis delta virus, the polyadenylation signal and cleavage site have been found to reside within elements of an RNA hairpin structure [79]. Other examples of this type of poly(A) hairpin were later found in a number of other human (and simian) lentiviruses and spumaviruses [80]. In HIV, the AAUAAA polyadenylation signal sequence is positioned within the terminal loop of the hairpin, with the CA cleavage site and part of the important downstream G/U-rich sequence located within the 3′ half of the base-paired duplex (fig. 5D) [79]. Therefore, although the details of the secondary structures surrounding the polyadenylation signals/sites of HDV and HIV differ slightly, there are general similarities between the two. Stability of the HIV poly(A) hairpin was found to influence a number of biological components of the virus in vivo [79, 80]. These studies have shown that increases in the level of polyadenylation, gene expression and viral replication resulted from reduction of the stability of the hairpin through the introduction of mismatches and bulges, whereas a decrease in those factors was seen by increasing the stability of the hairpin [79, 80]. In addition, there is in vitro evidence that the stability of the hairpin can limit the ability of the RNA to interact with polyadenylation factors, namely cleavage and polyadenylation specificity factor (CPSF) and cleavage stimulation factor (CstF) [81]. In essence, there was an inverse correlation between stability of the hairpin and binding of the factors [81].

In HIV, correct choice between two potential polyadenylation sites is necessary because of the repetitive nature of the HIV genome; this means that two poly(A) sites exist, one that is promoter proximal (the 5′ site) and one that is promoter distal (3′ site). Since polyadenylation at the 5′ site would be deleterious to the virus, the HIV genome has mechanisms to select for the 3′ site. One of these involves an upstream enhancer (USE) element found only upstream of the 3′ poly(A) site and required for efficient polyadenylation at this site both in vivo [82] and in vitro [83]. USE elements are generally U-rich but are otherwise rather amorphous, having no strong consensus sequence or length. Nevertheless, USE sequences have been found in a number of other viral and cellular RNAs (for example [84–86]). In the case of HIV, the USE has been shown to directly bind a subunit of CPSF [87]. A current model for polyadenylation in HIV is that the USE serves as an entry site for the binding of CPSF, which waits for the semistable poly(A) hairpin structure to open up through an equilibrium ‘breathing’ process, upon which CPSF gains access to the poly(A) signal and site to initiate the process of polyadenylation [81].

Due to the indistinct nature of the USE sequence, it is difficult to say whether a similar enhancer exists upstream of the polyadenylation signal in the HDV antigenomic RNA. No obvious highly U-rich domains stand out in this region of the sequence. However, just downstream of the proposed poly(A) hairpin is a U/C-rich J(–1/1) sequence that joins the poly(A) hairpin to the 5′ end of the antigenomic ribozyme. As discussed earlier, this sequence is one of the most highly conserved regions in the antigenomic RNA and was found to be important for polyadenylation of HDV mRNA [75]. In that study, the sequence downstream of this U/C-rich segment (i.e. the antigenomic ribozyme sequence) could be deleted with little effect on polyadenylation (87% polyadenlyation efficiency remained). However, deletion of an additional 17 nts (all of the U/C-rich stretch) resulted in nearly complete loss of polyadenylation (approximatey 4% remained). Of particular note, the poly(A) hairpin structure would be expected to remain intact in this last deletion, including the G/U-rich sequence discussed earlier. It may be possible that this downstream U/C-rich sequence is serving a role similar to the USE in the case of HIV. However, experiments are needed to test these ideas and define the exact role that this sequence is playing.

In at least one clear way, synthesis of the HDV antigenomic RNA is more complicated than that of the genomic RNA. This is because some of the antigenomic RNA is processed as message, a feat that must be distinct from the processing of replication products to form circular templates. The polyadenlylation site is upstream of the ribozyme cleavage site; therefore, although it is not necessarily the case that self-cleavage would prevent polyadenylation, it might affect it. However, it would clearly be detrimental to replication if polyadenylation were to occur on ribozyme-cleaved replication products since this would prevent subsequent circularization. Taking cues from the HIV model [81], we propose that P(–1) in the antigenomic sequence (the polyadenylation hairpin) could be involved in regulating polyadenylation. In this scenario, the P(–1) hairpin structure makes the RNA a relatively poor substrate for polyadenylation. However, the alternative pairing between the 3′ side of P(–1) and P2 of the ribozyme could make the polyadenylation signal and the poly(A) addition site more accessible, while at the same time preventing or delaying ribozyme activity (fig. 6). Under these conditions, the RNA would be cleaved at the polyadenylation site and poly(A) would be added. The exact nature of the alternative interaction is not critical in this model, and thus one might not expect to see a strict conservation of sequence and structure in this interaction. In this model, one structure would favor poly(A) addition and interfere with ribozyme activity, while the other would sequester the polyadenylation signals and favor ri-

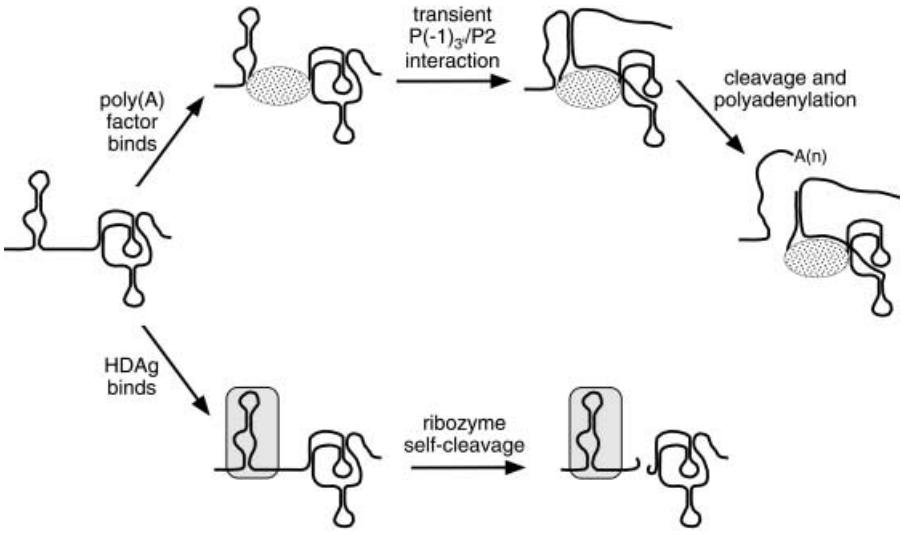

Figure 6. Hypothetical model for regulation of polyadenylation in HDV (see text for details).

bozyme activity (fig. 6). Binding of a polyadenlylation factor (CPSF?) to the U/C-rich sequence between P(–1) and the self-cleavage site might favor a transient $P(-1)_{3'}$/P2 interaction or directly inhibit the ribozyme (fig. 6). This last feature might also explain the slight differences observed in the sequences just 5′ to the cleavage sites of the genomic and antigenomic ribozymes (figs 4A, 5A). The presence of conserved purines in the genomic sequence could eliminate or reduce unwanted binding of a polyadenylation factor close to the ribozyme cleavage site. It has been reported that the HDAg can suppress polyadenylation [75, 88] and enhance ribozyme activity in cells [29]. In this model, binding of the HDAg to the poly(A) hairpin (and/or to part of the U/C-rich sequence) might block access of polyadenylation factors; thus, competition between the binding of HDAg and the polyadenylation factors has the potential to lead to the regulation of production of polyadenylated antigenomic RNA (fig. 6). Although there is no polyadenlylation of the genomic strand RNA, the HDAg could still function in promoting self-cleavage (and replication) of the genomic RNA by binding upstream of the cleavage site.

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L22066 (US-2); L22063 (Peru-1); U81989 (Ethiopia); X77627 (C. China); AF018077 (Taiwan); AF098261 (Canada); AF104263 (Taiwan); AF104264 (Taiwan); AF209859 (Taiwan); AJ000558 (C. Africa); M58629 (Nauru); M92448 (Taiwan); U19598 (Taiwan); U81988 (Somalia); M84917 (Lebanon1); D01075; M21012; M28267; M55042; NC_001653; X60193; X85253.

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