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Host RNA circles and the origin of hepatitis delta virus

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Abstract

Recent reports show that many cellular RNAs are processed to form circular species that are relatively abundant and resistant to host nucleases. In some cases, such circles actually bind host microRNAs. Such depletion of available microRNAs appears to have biological roles; for instance, in homeostasis and disease. These findings regarding host RNA circles support a speculative reappraisal of the origin and mode of replication of hepatitis delta virus, hepatitis delta virus (HDV), an agent with a small circular RNA genome; specifically, it is proposed that in hepatocytes infected with hepatitis B virus (HBV), some viral RNA species are processed to circular forms, which by a series of chance events lead to an RNA that can be both replicated by host enzymes and assembled, using HBV envelope proteins, to form particles some of which are infectious. Such a model also may provide some new insights into the potential pathogenic potential of HDV infections. In return, new insights into HDV might provide information leading to a better understanding of the roles of the host RNA circles.

Key words: Hepatitis B virus; Hepatitis delta virus; Circular RNA; Ribozymes; MicroRNA

Core tip: This article includes an evaluation of the hypothesis that in hepatocytes of patients chronically infected with hepatitis B virus, some viral RNA sequences can form small RNA circles, leading to the chance selection of one circle that becomes the progenitor of hepatitis delta virus.

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INTRODUCTION

Following the discovery of RNA splicing, a variety of circular RNAs were observed in animal cells. For example, there were reports for a tumor suppressor gene, deleted in colorectal cancer, in brain[1] and a sex-determining gene, SRY, in adult testes[2]. With time more examples of circular RNAs were reported, but there were consistent difficulties in proving that the species which were found, and which were never particularly abundant relative to mature spliced RNAs, had a function in cell biology. That is, that they were not just consequences of conversion from lariats, the canonical splicing intermediate, or worse, an artifact of experimental analysis. However, this picture has changed dramatically in the last three years. Many studies have now shown that circular species are actually abundant in cells[3-8]. Indeed, these RNAs are derived from a large fraction of all host RNA transcripts, both coding and noncoding RNA precursors. In some cases the circles are more abundant even than the canonical mature processed linear mRNA to which they are related. Numerous models have been proposed for how the circles are formed. Whatever their mechanism of formation, their circularity presumably confers metabolic stability against host RNases, which are predominantly exo- rather than endo-nucleases.

The finding of many and abundant host circular RNAs has led to searches for a biological role. One such role for an RNA circle, first found in the brain, is the ability to bind multiple copies of a host microRNA (miRNA)[8]. This so-called "sponge" effect[3,8] has been shown to have an essential role in homeostasis[7] and has also been speculated to play a role in human cancers[6]. No doubt more examples and even additional roles will be found for such RNA circles. The host RNA circles and their possible functions have already become the subject of numerous short commentary articles[9-13].

In 1986, three labs reported that the small RNA genome of hepatitis delta virus (HDV) has a circular conformation[14-16]. Replication of this viral RNA in infected cells leads to accumulation of antigenomes, which are also circular, and exact compliments of the genome. At the time of its discovery, the HDV RNA genome was considered to be unique relative to all other animal virus RNAs in terms of its small size, approximately 1700 nucleotides, and especially its circular conformation. Currently this is still true but it may not be for long, since we now know of the abundant host RNA circles, and as will be discussed, we might expect to soon see reports of other animal viruses producing small RNA circles.

The primary aim of this review is to reevaluate what we know about the origin and replication of HDV RNA in the light of the current knowledge of circular cellular RNAs, especially in terms of how they arise and their possible functions. Secondarily, the HDV studies might provide some useful insights for those studying host RNA circles.

HDV RNA CIRCLES

HDV was discovered in 1977 in patients with a more severe form of HBV infection[17]. In 1986, the RNA genome of HDV was shown to be circular in conformation[14-16]. During infection, as many as 300000 copies of HDV genomic RNA are transcribed per infected hepatocyte, with a somewhat lower amount of its exact complement, the antigenome[14]. The purified circular HDV RNAs were shown by electron microscopy to fold into an unbranched rod. Computer predictions support this interpretation[18] indicating potential intra-molecular base pairing for around 74% of all nucleotides. Assuming this folding also occurs in infected cells, it might be important in several ways, including RNA replication and stability. Nevertheless, extensive folding into a rod-like structure of purified HDV RNAs is considered to be relevant for binding by the delta antigen. Thus, it will be interesting to see for the recently described host RNA circles, whether intra-molecular base pairing occurs, and if so, whether it can have functional significance.

ORIGIN OF HDV

The increasing abundance of nucleotide sequences for viral and host genomes provides many opportunities for considering the origin of viral sequences[19,20], and in some cases viral sequences present as integrated DNA provide measures of the earlier times in history when a virus was present. Such "paleovirology" is relevant here in that it shows that relatives of duck hepatitis B virus, a relative of HBV, existed at least 20 million years ago[21] and maybe existed before HBV[22].

We and others have previously tried to ascertain the origin of HDV[23,24]. What has been grasped at, as a potentially key element, is the origin of viroids, RNAs that are infectious in plants[25]. Viroids have small genomes, 240-400 nucleotides in length, which can be circular in conformation. Viroids are thus even smaller than HDV and in addition, have no known protein coding capacity. Antigenomic RNA of HDV, in contrast, encodes one protein, the delta antigen. This protein, 195 amino acids in length, is essential for the accumulation of HDV RNA species in infected cells[26]. It is not translated from the circular antigenome, but from a less abundant linear mRNA, that is both 5'-capped and 3'-polyadenylated[27]. Thus, some authors have speculated that HDV might have arisen by a recombination between a viroid-like element and a host mRNA[28]. Candidates for such a host mRNA include DIPA[29], and more recently of CPEB3[20]. As will be explained, a better model can now be proposed.

HDV has been isolated from patients located in many parts of the world. Others have already compared the nucleotide sequences of eight recognized genotypes of HDV[30] in attempts to determine whether their relatedness indicates a possible common ancestor[31,32]. Some genotypes however differ by as much as 40% in nucleotide sequence, but all encode a delta antigen and all contain ribozyme domains, as discussed subsequently.

Since host RNA circles are abundant, diverse, and widespread, one might consider these as a potential source of an HDV RNA progenitor. However we do not have sufficient evidence to conclude that a cellular RNA is a likely origin of HDV, which requires not just a progenitor source of the delta antigen (see above) but, in addition, the ribozyme sequences, not so far found on host circular RNAs. Perhaps instead, HDV is derived from a viral RNA, and an obvious candidate for such a virus is HBV, as co-infection of the two viruses is required for HDV assembly. Several of the following factors might support this hypothetical chance event: (1) The life cycle of HDV depends on co-infection with HBV; (2) HBV causes chronic infections; at this time an estimated 350 million people are chronically infected[33]. This provides a huge reservoir from which HDV might have arisen (since HDV requires HBV as a helper); (3) Chronic infections can typically last 10-50 years in an individual[33]; this long time provides an increased window for a chance event; (4) The human liver contains about one trillion hepatocytes, and in a chronically infected individual most of these hepatocytes are infected. Such a large reservoir increases the opportunity for the chance production of an HDV-like RNA; (5) HBV replication involves transcription of RNAs from a covalently-closed circular double-stranded

DNA, cccDNA. The major processed HBV mRNAs that accumulate are not spliced. However, in studies of chronic infections, several studies have reported the presence of spliced RNAs[33]. (For duck hepatitis B virus, even during acute infections, one can readily detect spliced RNAs, some of which are derived from multimers, beyond even three times the length of the genome[33,34].) Thus, there are opportunities for aberrant HBV RNA splicing events within the hepatocytes of chronically infected individuals; and (6) In nature HDV replicates in hepatocytes. These cells are predominantly differentiated and non-dividing. As discussed further below, such an environment may be preferred for the accumulation of aberrantly processed RNA circles.

One or more of these six factors could increase the chance of producing an RNA circle that, like HDV, is replicated by host RNA polymerases. However, even then there will be the additional need of the circle (or its complement) to contain a coding region. This coding could be derived from HBV sequences or alternatively, by an event such as trans-splicing[5] or perhaps transcription from an integrated HBV DNA sequence, to incorporate host RNA sequences that might be expressed as a translatable RNA, to produce a protein that will support accumulation of new transcripts and the release of some of genomic RNA, *via* interaction with the envelope proteins produced and released from HBV infected cells.

HBV envelope proteins encapsidate HBV (and HDV) nucleoscapids. Importantly, they are produced in vast excess of the amount needed to produce HBV, with the excess being released as distinctive "surface antigen" particles. It is estimated that surface antigen particles may be released in an up to 100000-fold of excess of virus[33]. This excess would favor the chance for release of a particle that contains an HDV RNA and is able to replicate RNA delivered, by HBV envelope proteins, to a new hepatocyte. New rounds of virus production would be ensured if the target cell is already infected by HBV, which is likely to be the case in HBV patients.

In terms of the properties demanded of the delta antigen itself, it is worth noting that this protein is highly basic and readily interacts with nucleic acids[35]. Furthermore, it has recently been shown to have a high level of intrinsic disorder[35]. Like other viral proteins also known to have high intrinsic disorder, the delta antigen readily multimerizes and it forms association with many host proteins[36], as well as with a specific HBV envelope protein domain.

While this broad hypothesis regarding the origin of HDV might seem plausible at this time, there are negative aspects that have to be faced. The most significant of these is that no sequence homology has yet been detected between HDV and any strain of HBV. However, it could be that the HDV sequence has evolved so much that an original homology is no longer apparent. Error-prone RNA-directed RNA transcription could be a major source of nucleotide sequence divergence[20]. In addition, the folding of RNA molecules might have provided multiple targets for post-transcriptional RNA editing, such as by adenosine deaminases, which would be especially important over time if that RNA was able to replicate. Interestingly, site-specific editing by an adenosine deaminase already plays an essential role in the HDV life cycle, leading to production of a modified form of delta antigen that has an essential role in genome packing into virions[37].

A summary of the steps involved in the above hypothesis is presented in Table 1.

With regard to tracing the origins of HDV, it is important to note that the eight known HDV genotypes show extensive nucleotide sequence diversity, with an extreme of 40% mismatch between two genotypes[30]. Such extensive divergence might even allow the further speculation that there may be not one but multiple distinct origins of HDV arising from HBV-infected hepatocytes. That is, HDV is not a "relic of an RNA world"[38,39] or even a virus only as old as the hepadnaviruses[21] but is, instead, a satellite of HBV that has arisen much more recently and possibly several times through chance splicing events linked to individuals chronically infected with HBV.

The hypothesis regarding the origin of HDV from HBV infected hepatocytes, together with the facts concerning its replication, allow some additional speculations.

First, virus infections other than HBV may also produce RNA circles. A promising example might be polyoma virus, where double-stranded DNA circles act as templates for multiple RNA transcripts, some of which are up to several times unit-length, and all subject to RNA splicing events[40].

Second, it is possible that some host RNA circles, and even hypothetical non-HDV viral circles, can be replicated by host RNA polymerases. RNA-dependent RNA synthesis does not have to be as efficient as that of HDV. Even limited RNA-directed RNA transcription could enhance effects of the circular RNAs on the host cell; for example, it would increase the "sponge" effect on specific host miRNAs that are able to bind to the RNAs[3,8].

HDV RIBOZYMES AND HOST HDV-LIKE RIBOZYMES

Both the genomic and antigenomic RNA of HDV contain a single small sequence that will function *in vitro*, as a ribozyme[41-43]. Such activities have suggested relatedness to examples of self-splicing of host RNAs, but to date, this or a connection to canonical splicing has not been confirmed. The two HDV ribozymes have sequence and predicted structure relatedness to each other, which has been linked to the rod-like folding of the genome and antigenome[42-44]. *In vitro*, HDV ribozyme activity can be reversed to achieve self-ligation[45]. However, others have argued that *in vivo* this end joining reaction, like that involved in tRNA precursor splicing, is carried out by a cellular ligase, rather than a viral ribozyme[46].

The HDV self-cleaving ribozymes were first thought

Table 1 Hypothesis for the origin of hepatitis delta virus

An acute hepatitis B virus (HBV) infection becomes chronic, spreading to virtually all the hepatocytes in the liver
HBV replication also involves transcription of many viral sequences some of which undergo splicing, with the potential to form stable RNA circles
A rare RNA circle is selected both because it has the rare capacity to undergo RNA directed replication using host polymerase(s) and because it expresses a short protein that is both intrinsically disordered (favoring multimerization) and positively-charged (favoring RNA binding)
Further replication and accumulation selects for increased efficiency, as facilitated by the encoded binding protein, which becomes the small delta antigen. The replicating RNA sequence undergoes many nucleotide changes, rendering it unrecognizable relative to HBV sequences. Such changes could be introduced by misincorporation during transcription and post-transcriptional RNA editing events
A site-specific post-transcriptional modification of the RNA by an RNA editing enzyme leads to translation of a C-terminal elongated second form of the protein, the large delta antigen which undergoes farnesylation. This farnesylation facilitates the binding of the hepatitis delta virus (HDV) ribonucleoprotein complexes to HBV envelope proteins, leading to the release of virus particles that can infect susceptible hepatocytes and undergo further rounds of replication. This, in the presence of HBV co-infection, leads to the release of more HDV

As listed above the hypothesis involves multiple steps, several of which would be rare events that have to be selected for. However, as separately enumerated in the text, there are other parameters that could favor such events.

to be quite unique relative to other known ribozymes, both in sequence and predicted folding. Even after experimental determination of the tertiary structure of the HDV ribozymes[47], they seemed unique relative to previously determined structures of other ribozymes. However, when the structure and folding were used in order to search for "HDV-like ribozymes", one was found in the intron of a host mRNA, CPEB3[48], and then many, many, more were found, across all kingdoms of life except Archaea[49,50]. In several cases these putative ribozymes were tested *in vitro* and proven to be functional[49]. In addition, it has been noted that some HDV-like ribozymes are so located that they may process transcripts of non-LTR retrotransposons, and possibly facilitate translation of such RNAs[50,51]. In apparent contrast to this, the ribozyme located on HDV antigenomic RNA is located downstream rather than upstream of the open reading frame for the delta antigen. This downstream location is immediately preceded by sequence signals, such as AAUAAA, as are used in typical polyadenylation of host mRNAs[52].

A weakness of the present hypothesis of HDV origin *via* HBV sequences (as summarized in Table 1) is that it does not explain the origin of the two ribozymes. The model implies they may have been associated solely with expressed HBV sequences, but a modification is that variant splicing of expressed HBV sequences (possibly as transcribed from integrated HBV sequences) created a link to host sequences, leading to an RNA circle with associated HDV-like ribozyme sequences and even the protein encoding region that becomes the small delta antigen.

It should be mentioned that *in vitro* studies have demonstrated the ability of HDV RNAs to bind proteins some of which are involved in RNA processing[53]. Examples include the splicing factor, ASF/SF2[54] and the polypyrimidine tract-binding protein-associated splicing factor, PSF[55]. In terms of the latter finding, others have previously noted that the HDV RNAs have multiple polypyrimidine tracts[56].

While this may seem supportive of a splicing hypothesis, some caution is still needed since many host proteins not involved with splicing have been reported as binding to HDV RNAs; examples include eEF1A1, p54(nrb), hnRNP-L, and GAPDH[53,54,57].

REPLICATION/TRANSCRIPTION OF HDV RNAS

For many years attempts have been made to understand how HDV RNAs are transcribed from RNA templates, and whether such transcription could be reconstructed *in vitro* with host nuclear extracts or even purified host polymerases. There is agreement that host RNA polymerase Ⅱ is redirected to act on an HDV RNA template[58]. In addition, there are data supporting a model of how RNA polymerase Ⅱ might act to transcribe RNA from an HDV RNA template[59]. The participation of this host enzyme normally used for the transcription from host DNA (and of course HBV cccDNA) templates leading to mRNA species, maybe provides circumstantial evidence for the above-described hypothesis concerning the origin of HDV. That is, host RNA polymerase Ⅱ transcripts are the ones destined for processing events that include splicing and polyadenylation, and as now understood, RNA circle formation.

Nevertheless, there remains controversy as to whether or not HDV RNA-directed RNA transcription also makes use of another host RNA polymerase[60]. There is also controversy as to how aspects of HDV RNA-directed transcription deduced from *in vitro* transcription assays reflect what happens *in vivo*. For example, while polymerase Ⅱ has been found to bind the ends of HDV rod-like RNA sequences[61], subsequent studies show that so also do polymerases Ⅰ and Ⅲ[62].

At the present time, it is not entirely clear how transcription from HDV genomic and antigenomic RNA templates is initiated. One clue comes from the observation that HDV replication leads to the accumulation of a less than full-length linear RNA (it is up to 500-times less abundant than the accumulated genomic RNA circles[14,52]). This RNA has a 5'-cap and a 3'-polyadenylation. It contains the open reading frame for the delta antigen, the one protein of HDV, and is considered the relevant mRNA. In other words, it looks like a typical mRNA produced by RNA polymerase Ⅱ. Thus, it is assumed that its 5'-end, which maps near one end of the rod-like genomic RNA template, might be a site of initiation and

therefore help define a promoter element on the HDV RNA template.

However there must also be at least one additional promoter site on the antigenomic RNA template for transcription of genomic RNA. Indeed, there may be additional, possibly multiple initiation sites on both RNAs; after all, on circular RNA templates, transcripts (by whatever RNA polymerase, not necessarily polymerase Ⅱ) that can elongate to beyond greater than unit-length, can have more than one ribozyme site, and could thus be processed to unit-length, and subsequently ligated to form new circles. This concept has for quite some time been included in a somewhat vague rolling-circle model of HDV replication[63]. Maybe, theoretically, replication of host RNA circles[9] might occur by a similar process.

No one has yet determined if HDV RNA circles, like host RNA circles, bind host miRNAs. If such binding does occur, this might lead to primed transcription of viral transcripts, some of which might be processed to unit length linear and then circular RNAs. As discussed below, circular RNAs, once formed, may have unique advantages, especially in terms of stability.

RNA CIRCLES AND THEIR RESISTANCE TO EXO-NUCLEASES

For some time it has been known that in cell extracts, RNA species in a circular conformation have much greater stability than the corresponding linear species[64]. The interpretation is that host nucleases are primarily exo- rather than endo-nucleases. This advantage has been ascribed to the naturally occurring HDV circular RNAs and more recently to the host RNA circles. One recent study confirmed the stability of the host RNA circles relative to other linear RNA species[5].

In addition, it has been noted that host RNA circle stability increases with decreasing size. Earlier studies established this relationship for deleted forms of the HDV RNA[65]. In addition, while the presence of the delta antigen enhanced the accumulation of processed full-length HDV RNA circles, the presence of delta antigen became irrelevant for RNA species that were about four times shorter[65]. Further study revealed that small and large circles could only accumulate when the ribozyme domain present on the circle was able to make intra-molecular base pairings that have been presumed to force the ribozyme into an alternative, inactive conformation[66,67].

The susceptibility of RNA circles to degradation *via* targeting with small interfering RNA, siRNA, might be variable. One study of individual host RNA circles demonstrated susceptibility[5]. In contrast, a study of HDV RNAs, both *in vivo* and *in vitro*, detected no degradation associated with DICER activity[68].

ROLE(S) OF THE SMALL DELTA ANTIGEN

A form of the delta antigen, 195 amino acids long, is essential for the accumulation of HDV RNAs in an infected cell[26]. Later it was found that a second form, 19 amino acids longer at the C-terminus, arises as a consequence of site-directed RNA editing of antigenomic RNA during replication[69]. This so-called large form of delta antigen is farnesylated near its novel C-terminus. Although it cannot support HDV RNA accumulation and is a dominant-negative inhibitor of replication supported by the small form, it is essential for the formation of enveloped virions *via* interaction with the HBV envelope proteins[70].

While the small delta antigen is essential for the accumulation of processed HDV RNAs it is not yet clear in what manner(s) it is essential. For example, it is controversial as to whether the protein has a direct or even indirect role in the transcription process[71,72]. Another example is whether or not the small delta antigen is needed as a "chaperone" for the cleavage and processing by the HDV ribozyme[65,73].

PATHOGENIC POTENTIAL OF HDV

While it is clear that HDV/HBV co-infection is more damaging than one with HBV alone, the mechanism(s) involved are still controversial[74]. Experimentally it is possible to set up situations where HDV is replicating its genome in the absence of HBV. It is relevant to the present discussion that two different outcomes might arise from such mono-infections.

First, in hepatocytes that are well-differentiated and non-dividing, it seems that HDV replication, *per se*, is not pathogenic. HDV to a small extent will infect mouse hepatocytes, *in vivo*, and the clearance of such infections takes 5-15 d, independent of the presence or absence of a host immune response[75]. Similarly, HDV will infect woodchuck hepatocytes, and even after 5 wk infected cells remain from which HDV can be rescued by a superinfection with woodchuck hepatitis virus[76]. And of most relevance, HDV can infect human hepatocytes maintained by transplantation into mice in which the resident mouse hepatocytes have been largely destroyed. These HDV infected cells do not decrease with time and even after 6 wk, superinfection with HBV rescues HDV, leading to spread of HDV to human hepatocytes that were not initially infected by HDV[77].

Second, consider the following model system[78]. A human cell line, HEK293, was transfected with an HDV RNA, one mutated so that the delta antigen cannot be expressed. This cell line also contained a stably integrated cDNA copy of the HDV antigen, with expression inducible by tetracycline. Even without induction, the amount of delta antigen produced was sufficient to maintain low levels of HDV RNA replication and accumulation for at least three years. However, when the antigen was induced, the amount of HDV RNA accumulation increased up to 40-fold, and within 48 h led to cell cycle arrest and detachment of the cells from monolayer culture. More importantly, when host individual host RNA levels were assayed, even by 24 h the levels of more than 1000

host genes were altered by at least 2-fold (with > 95% confidence level). In control cells expressing only the increased amount of delta antigen, the number of host mRNAs changed was ten times less[74]. Thus, in some cells high level replication and accumulation of HDV RNAs might have a significant impact on cell gene expression and viability.

From the above studies, we might conclude that HDV RNA replication and accumulation is more damaging when the cells are dividing than when they are non-dividing (as is typically the case with human hepatocytes). If this interpretation is correct, one of many inferences could be that host RNA circles can accumulate to a greater extent and with less deleterious effect in differentiated non-dividing cells, such as hepatocytes and yet, when the cells are induced to divide (*e.g.*, to replace hepatocytes destroyed by the antiviral immune response), RNA circles might perturb host RNA levels and lead to decreased viability of the infected cells.

Interestingly, in HEK293 cells, Memczak *et al*[8] have described the accumulation of a specific 1500 nt circular host RNA that binds multiple copies of a specific host miRNA, and has a regulatory role. Changes in the amount of this RNA or of the miRNA that binds to it, had profound effects on the levels of many host RNA species.

CONCLUSION

New realizations concerning circular forms of host RNAs have provoked this re-examination of aspects of HDV, especially its origin and the mode of replication, and also suggested new insights into potential HDV-associated pathogenesis. However, it should also be acknowledged that HDV studies might have a reciprocal relevance to the host circular RNAs, such as how they arise and affect the host cell. And, although not considered here, there remains a need for a similar re-examination of the replicating small circular RNAs of plants, that is, the viroids and virusoids.

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