Self-Ligating RNA Sequences on the Antigenome of Human Hepatitis Delta Virus

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A 179-base fragment of RNA from the 1,679-base antigenome of hepatitis delta virus can not only self-cleave but, when the ends of the resultant fragments are brought into apposition by base pairing to another RNA, also self-ligate. Thus, processing events needed for genome replication in vivo may be strictly RNA mediated.

The single-stranded RNA genome of hepatitis delta virus (HDV) is unusual in that it is circular in conformation and is able to fold on itself by base pairing to form an unbranched rod structure (3, 8, 15). Inside infected cells, not only is this genomic RNA present but also its complement, the so-called antigenomic RNA. These RNAs occur mostly as single-stranded unit-length species of which about half are in a circular as opposed to a linear conformation (3). Also present are minor amounts of multimeric lengths of genomic and antigenomic RNAs (3). As reviewed elsewhere (J. Taylor, L. Sharmeen, M. Kuo, and G. Dinter-Gottlieb, UCLA Symp. Mol. Cell. Biol., in press), these characteristics of the HDV genome and its replicative RNAs are very similar to what has already been seen for certain infectious RNAs of plants (7). Thus, it is likely that HDV genome replication, like that of the plant RNAs, may involve some kind of rolling-circle process. Models of such processes usually invoke the synthesis of RNA species that, at least transiently, are larger than unit length and thus require subsequent cleavage and ligation events to make new unit-length circular RNAs (1). Some of the infectious plant RNAs have been shown to be able to carry out such processing in vitro in the absence of proteins (2, 4-6, 10). Recently, we have reported that both the genomic and the antigenomic RNAs of HDV can self-cleave in a site-specific and efficient manner (9, 12). We now report that the antigenomic RNA of HDV can be induced to carry out a self-ligation event, again with high efficiency and specificity, in the absence of any proteins.

There were three major concepts that provided the incentive and the rationale for the self-ligation experiments described in this report. The first was that there was a relevant precedent for self-ligation, namely, the satellite of tobacco ringspot virus (2, 10). The second was our speculation that sequences from the opposite side of the rod might be not only inhibitors of self-cleavage but also promoters of self-ligation (9). The third concept was that Usher and McHale had demonstrated the ability of hexa-adenylic acid to self-ligate and make 12-mers and even 18-mers (13, 14). This process is strictly dependent on two things. First, the ends of the hexamer must be a 5'-OH and a cyclic 2', 3'-monophosphate. Second, polyuridylic acid must be present, presumably to act as a backbone to bring the ends of the hexamers into apposition.

Our basic experimental hypothesis to test for self-ligation with HDV RNA sequences is represented in steps 1 to 3 of

Fig. 1. We synthesized in vitro a small antigenomic HDV RNA that spanned the self-cleavage site at position 901/900. (Our notation for the 1,679-base genome is as described by Kuo et al. [8].) This RNA was then fully self-cleaved in vitro. We also synthesized a small antigenomic RNA from the opposite side of the rod. This RNA was then hybridized to the two self-cleavage fragments and was subjected to the

FIG. 1. Self-ligation reaction. (a) Summary of the strategy. A uniformly labeled 179-base antigenomic RNA (from positions 963 [SalI] to 785 [XbaI]), designated A, was submitted to self-cleavage (9, 12) to produce a 116-base 3' fragment, designated B, and a 63-base 5' fragment, designated C. These fragments were then hybridized (for 1 h at 50°C in 600 mM NaCl-40 mM Tris-2 mM EDTA) to an unlabeled 136-base antigenomic RNA (from positions 785 [XbaI] to 650 [PstI]), designated D. As a consequence of the self-ligation conditions, some of the fragments B and C religated to produce A. To test the ability of the newly formed A to self-cleave, the ligation mixture was denatured and band A was gel purified away from the other RNAs before being tested. (b) Direct quantitation with a Radioanalytic Imaging system (AMBIS, San Diego, Calif.) of the gel analysis (12) of ligations carried out with the following molar ratios of D to labeled B and C: panel 1, 0; panel 2, 1; panel 3, 10.

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TABLE 1. Dependence of self-ligation on relative concentration of backbone RNA

RNA D/labeled fragments B and C ^a	% Ligation ^b
0	<2
0.3	<2
1	6
3	21
10	44
20	28

^a Molar ratio.^b Self-ligation and quantitation were carried out as described in the legend to Fig. 1.

self-ligation conditions used by Usher and McHale (14), namely, 2 days at 4°C in 1 M ethylenediamine. Ligation was then monitored, with results as shown in Fig. 1. Self-cleavage fragments B and C self-ligated to produce an RNA species with a mobility the same as that of the original RNA A if unlabeled RNA D was present (panels 2 and 3). No ligation was observed when RNA D was omitted (panel 1). The amount of ligation was increased when the molar amount of RNA D, relative to that of RNAs B and C, was increased (panel 3 relative to panel 2). Panel 3 corresponded to a ligation efficiency of 44%. When we considered a range of values for the ratio of RNA D to RNAs B and C, the extent of ligation increased from zero and leveled off when the ratio was as high as 3- to 20-fold, possibly peaking at 10-fold (Table 1). We also examined the effect on ligation of several other variables (Table 2). The RNA that had ligated could not be self-cleaved (data not shown) by conditions that for uncleaved RNA are normally >99% efficient (9). However, after we used gel electrophoresis to purify the ligated species, A, away from species D, more than 75% of the RNA could be cleaved (data not shown). The electrophoretic mobilities of the released fragments showed that recleavage occurred at approximately the same site. However, there was an unexpected result when we examined the ability of reverse transcriptase to extend an end-labeled DNA primer and read through an RNA template with a ligated site

TABLE 2. Variables affecting self-ligation efficiency

Variable	% Ligation ^a
Prehybridization temp (°C)	
68	19
50 (standard)	10
25	1
4	<2
Ligation temp (°C)	
4 (standard)	8
25	17
Ligation time	
14 days	20
45 h (standard)	8
4.5 h	<1
0.17 h	<1
Ligation buffer	
1 M Ethylenediamine (standard)	12
100 mM MgCl ₂	3
T4 DNA ligase buffer ^b	2

^a Ligation of B and C in the presence of D, as summarized in Table 1.^b Ligation buffer contained 10 mM MgCl₂-50 mM Tris (pH 7.6)-5% polyethylene glycol-1 mM ATP-1 mM dithiothreitol.

FIG. 2. Primer extension on RNA before and after self-ligation. Labeled RNA fragments B and C were submitted to self-ligation as described for panel 3 of Fig. 1b. We then gel purified both the ligated RNA (lanes 1, 2, and 3) and, as a control, fragment A RNA that had never been cleaved (lanes 4 and 5). These two RNAs were used in primer extension either without (lanes 1 and 4) or with (lanes 2 and 5) a subsequent self-cleavage treatment. Primer extensions were carried out as described previously (9, 12) and in the text, and the products were separated on a DNA-sequencing gel as shown in panel A. Lane 3 is a mixture of lanes 1 and 2. The primer extension products P, Q, and R are explained in panel B.

relative to an RNA that had not previously undergone a ligation event. Primer extension of the control RNA that had never been ligated (Fig. 2A, lane 4) yielded predominantly one band, R. When this RNA was submitted to self-cleavage prior to primer extension (Fig. 2A, lane 5), the amount of band R was reduced and there was a corresponding increase of a shorter species, band P. As summarized in Fig. 2B, from our previous studies (9, 12), we knew that band R represents primer extension out to the 5' end of the RNA, whereas band P represents extension to the self-cleavage site. We next examined the self-ligated RNA. The ligated RNA yielded not only the expected band R, but also an almost equivalent amount of a novel band, Q (Fig. 2A, lane 1). The electrophoretic mobility of band Q indicated that it was one nucleotide longer than band P. When the ligated RNA was recleaved prior to primer extension (lane 2), the major differences were the reduction of band R and the appearance of band P.

Our interpretation is that prior to recleavage, only about half of the template RNA can be read through to the 5' end by reverse transcriptase. The other half apparently terminates, for reasons not yet clear, at 1 base beyond the self-ligation site. After recleavage, there is the additional appearance of molecules that have self-cleaved at the expected site. Thus, we interpret that our self-ligation reaction produces approximately equal amounts of two different types of junctions. Only one of these, like natural RNA, is able to be read through by reverse transcriptase and able to be self-cleaved at a specific site. On the basis of the work of others (2, 10, 11, 14), we speculate that one junction is 3' to 5' and the other is 2' to 5'. The ligation we have seen in vitro with three RNAs probably occurs in vivo as part of a single unit-length linear RNA and yields circles (Taylor et al., in press). Such a reaction is likely to be much more efficient (actually concentration independent) and more specific (probably sterically blocking the creation of the unnatural 2' to 5' linkage).

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