Alternative hammerhead structures in the self-cleavage of avocado sunblotch viroid RNAs

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ABSTRACT

The plus and minus RNAs of the 247 nt avocado sunblotch viroid (ASBV) undergo site specific RNA self-cleavage reactions *in vitro*. As with several other self-cleaving RNAs, we proposed hammerhead secondary structures for the sequence around the site of self-cleavage of both RNAs. We have shown previously that, during transcription of a dimeric plus ASBV RNA, a double-hammerhead structure formed and was necessary for self-cleavage. Here, we show that the purified full-length dimeric plus RNA, when incubated under our standard self-cleavage conditions, also self-cleaved by a double-hammerhead structure. In contrast, a dimeric minus ASBV RNA self-cleaved by a double-hammerhead structure during transcription, but by a single-hammerhead structure after purification. This illustrates the importance of the pathway of folding of the RNA in determining which active self-cleaving structure is formed.

INTRODUCTION

In vitro site-specific RNA self-cleavage reactions occur in a variety of low molecular weight single-stranded RNAs (Refs. 1–12). This self-cleavage reaction is believed to be important *in vivo* in the replication of the certain pathogenic RNAs by a rolling circle mechanism (13,14), in which multimeric RNAs undergo site specific self-cleavage to generate monomer units (15).

These *in vitro* self-cleavage reactions occur in the absence of proteins and require only the presence of a divalent cation, such as Mg^{2+}, and around neutral pH conditions to yield 5′-hydroxyl and 2′,3′ cyclic phosphodiester termini. In one case the self-cleavage reaction is reversible (4). There appear to be four types of RNA structures which mediate self-cleavage (Refs. 5,7–10,12,16,17).

The best characterised type of RNA self-cleavage is that mediated by the hammerhead structure (5), which occurs, or is predicted to occur, in 11 unique species of naturally occurring RNAs (5,7,15). The hammerhead structures consist of three base-paired stems enclosing inner single-stranded regions and include 13 conserved bases (5,15,18–20). Not all of the nucleotides conserved *in vivo* are essential for the *in vitro* self-cleavage reaction (7,21–25). Presumably, in the presence of a divalent cation such as Mg^{2+}, the hammerhead secondary structure forms an active tertiary complex that lowers the activation energy sufficiently and specifically at the internucleotide bond of the cleavage site to allow the phosphoryl transfer of the self-cleavage reaction.

The hammerhead structures of most of the RNAs appear stable. However, the hammerhead structures of plus and minus ASBV and newt RNAs appear theoretically unstable due to the presence of weak stem IIIs with sterically constraining loops (Figure 1; Refs. 1,18,20). More stable secondary structures have been proposed for these RNAs which involve the interaction of two hammerhead sequences to form double-hammerhead structures (Figure 1; Ref. 2), which have theoretically more stable stem IIIs while maintaining the other features of the single-hammerhead.

Stability of the single-hammerhead stem III has been found to be an important factor in determining whether self-cleavage occurs by a single- or a double-hammerhead structure. Using short RNAs containing sequences based on, but not identical to, the self-cleaving newt sequence, Sheldon and Symons (26) investigated the requirements for the single-hammerhead mediated reaction versus the double-hammerhead reaction in terms of the stability of the single-hammerhead stem III. It was determined that the minimum requirement for single-hammerhead self-cleavage in this system was a stem III of 2 base-pairs with a loop of 4 bases, or a stem III of three base-pairs with a loop of three bases. A consequence of this work is that it would be expected that the minus RNA of ASBV should be capable of self-cleavage by a single-hammerhead structure, but no published data are available. Evidence that two short RNA oligonucleotides (19 nt and 23 nt) based on the minus ASBV hammerhead sequence self-cleaved by a single-hammerhead structure (27), further indicated that minus ASBV RNA should be able to self-cleave as a single-hammerhead.

In this paper, the work by Forster *et al.* (2) on the double-hammerhead mediated self-cleavage during transcription of a 528 nt dimeric plus ASBV RNA was extended to include analysis

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of self-cleavage of both plus and minus dimeric RNA transcripts during the transcription reaction as well as with the purified full-length RNAs. The results demonstrate that the plus RNA is capable of self-cleavage only by a double-hammerhead structure under the conditions, whereas the minus RNA undergoes self-cleavage by a double-hammerhead structure during transcription and by a single-hammerhead structure after purification of the RNA and incubation under our standard self-cleavage conditions. They also demonstrate the importance of the pathway of folding of RNA in determining single- or double-hammerhead self-cleavage reaction in minus ASBV RNAs.

MATERIALS AND METHODS

Reagents

SP6 RNA polymerase, Klenow DNA polymerase I, T4 polynucleotide kinase, T4 DNA ligase, α-^{32}P-UTP and α-^{32}P-dATP were from Bresatec, Adelaide, calf intestinal alkaline phosphatase from Boehringer Mannheim, and restriction enzymes from Pharmacia and New England Biolabs.

Plasmid constructions of ASBV cDNA clones

Plus dimeric Sau3A clones in the plasmid vector pSP64 (Figure 2A). The construction of the plasmids containing a dimer of the wild-type ASBV cDNA cloned at the Sau3A position (terminal ASBV nucleotides 153 and 154) in the plus orientation, in the transcription vector pSP64 has been described (1). The construction of similar clones that are mutant in either or both of the plus ASBV hammerhead sequences (the conserved GAAA-C sequence mutated to GAAC) have also been described (2).

Dimeric BstNI clones in the plasmid vector pGem1 (Figure 3A). An ASBV cDNA monomer cloned at the Sau3A site of phage M13mp93 DNA was used for oligonucleotide-directed mutagenesis, essentially as described by Zoller and Smith (28), to mutate the GAAAC sequence in the minus hammerhead sequence to GAAC. An existing Sau3A dimeric clone in the minus orientation in the plasmid pSP65 (1) was digested at the junction of the two ASBV monomers with BclI and the mutant Sau3A monomer cDNA ligated in (BclI and Sau3A are compatible). This created a trimeric Sau3A clone in which the middle Sau3A monomeric unit was mutated. Digestion of this clone with BstNI gave a mixture of two monomeric fragments, with terminal nucleotides 51 and 52, one with wild-type sequence and one containing the mutation. These monomeric fragments have a one base 5′ overhang and were end-filled using the Klenow fragment of DNA polymerase I. Dimeric fragments resulting from ligation of mutant and wild-type BstNI monomeric fragments in a 1:1 ratio were ligated into dephosphorylated SmaI digested pGem1 vector. Clones wild-type in both minus hammerhead sequences, or mutant (conserved GAAAC sequence mutated to GAAC) in either or both were identified by subcloning into M13 followed by dideoxy sequencing (29). Note that the end-filling step destroyed the BstNI site (CC/AGG) between the ligated monomers and created a StyI site (C/CAAGG) (Figure 3).

In vitro transcription from linearized plasmid templates and isolation of RNA transcripts

Clones were digested with the appropriate restriction enzyme (as indicated in the figures) and transcribed with SP6 RNA polymerase. Transcription reactions contained 0.1 μg/μl DNA template, 0.5 U/μl SP6 RNA polymerase, 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 0.1 μg/μl bovine serum albumin, 10 mM DTT, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.025 mM UTP and 2 μCi/μl α-^{32}P-UTP, and were incubated at 37°C for 1 h. RNA transcripts were separated on a 7 M urea, 5% polyacrylamide gel, and full-length transcripts were excised from the gel and eluted as described by Forster and Symons (5).

In vitro self-cleavage of isolated RNAs

Standard self-cleavage conditions: gel purified full-length RNA transcripts in 1 mM EDTA, pH 6, were heated at 80°C for 1 min, snap-cooled on ice and then ice cold buffer was added to a final concentration of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.5 mM EDTA. The reactions were incubated at 37°C for 1 h, and terminated by the addition of EDTA to 10 mM and an equal volume of formamide. Products were resolved on a 7 M urea, 5% polyacrylamide gel and identified by autoradiography.

RESULTS

Dimeric Plus ASBV RNA Self-Cleaves Only By a Double-Hammerhead Structure Both During Transcription and After Purification

Dimeric plus ASBV RNA transcripts, generated *in vitro* by SP6 RNA polymerase transcription of the dimeric Sau3A cDNA template (Figure 2A), have previously been shown to self-cleave, during the transcription reaction, by a double-hammerhead structure (2). The requirement for a double-hammerhead structure was demonstrated by the use of single-base mutations in which the conserved GAAAC sequences just 5′ to the self-cleavage sites, SC-1 and SC-2 (Figures 1, 2A), were mutated to GAAC (deletion of one A residue) either separately, or together to give a double mutant. Abolishment of self-cleavage at the self-

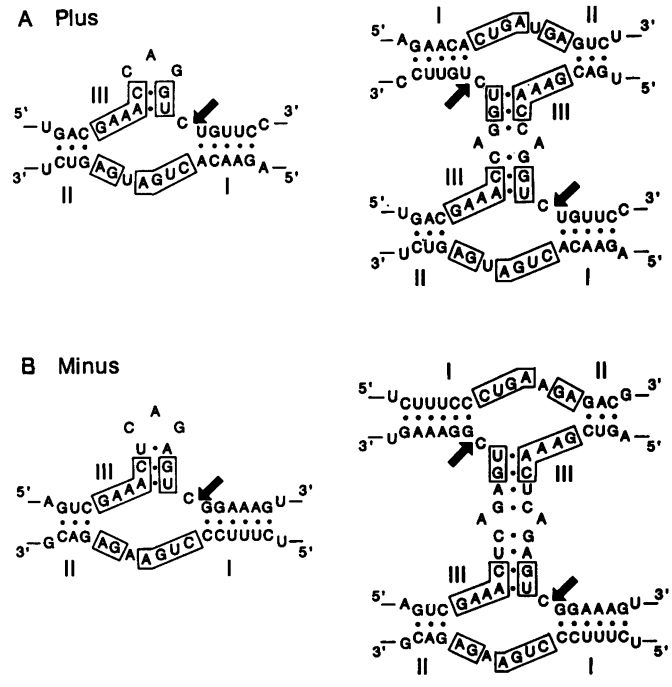

Figure 1. Single- and double-hammerhead structures of the self-cleaving RNAs of plus (A) and minus (B) ASBV (2). Base-paired stems are numbered I to III after Forster and Symons (5), sites of cleavage are indicated by arrows and nucleotides conserved between all self-cleaving RNAs (5,7,15,20) are boxed.

cleavage site over 250 bases away from the mutated GAAAC sequence was indicative of double-hammerhead mediated self-cleavage. Inhibition of self-cleavage at the self-cleavage site just 3′ to the mutated GAAAC sequence would have been indicative of single-hammerhead mediated self-cleavage (2).

During the transcription reaction, the efficiency of self-cleavage of the plus dimeric RNAs mutated at one site was about 50% (Ref. 2; Figure 2B, lanes 4,6); presumably the residual uncleaved RNA was folded into inactive conformations that did not permit self-cleavage at that site. We have now purified the full-length mutated plus RNAs and analysed them under our standard self-cleavage conditions (Materials and Methods). The self-cleavage products obtained were the same as those obtained during the transcription reaction for both RNAs in which the first GAAAC sequence (GAAAC-(A); Figure 2A) only was mutated (Figure 2B, lanes 6,7) and in which the second GAAAC sequence (GAAAC-(B); Figure 2A) only was mutated (Figure 2B, lanes 4,5); indicating that self-cleavage occurred by a double-hammerhead structure under these conditions also. As expected, self-cleavage was abolished at both sites in purified RNA with both GAAAC sequences mutated (Figure 2B, lane 3). Therefore, the dimeric plus RNA self-cleaved by a double-hammerhead structure both during the transcription reaction and with the isolated full-length RNA.

Dimeric Minus ASBV RNA Self-Cleaves During Transcription By a Double-Hammerhead Structure

Minus dimeric ASBV RNA transcripts generated from a Sau3A clone have been previously shown to undergo self-cleavage between nt C70 and G69 both during transcription and after purification (1); however, it was not determined whether self-cleavage occurred by a double- or a single-hammerhead structure. The single-hammerhead structure of minus ASBV has greater theoretical stability than the plus single-hammerhead structure, due to the presence of an extra base-pair in stem III (Figure 1). To investigate whether a single- or a double-hammerhead structure is involved, site-directed mutations in the minus hammerhead structure, analogous to those described above for the plus hammerhead structure, were constructed.

Wild-type dimeric minus Sau3A transcripts have been previously shown to self-cleave with different efficiencies at the two self-cleavage sites (1). This, apparently, was due to the effect of the cloning site and/or the presence of the vector sequence on the folding of the RNA transcripts during the transcription reaction. As transcripts from a wild-type BstNI dimeric clone self-cleaved with equal efficiency at both self-cleavage sites (Figure 3A, SC-1 and SC-2; Figure 3B, lane 1), BstNI clones were chosen for the construction of the mutant clones.

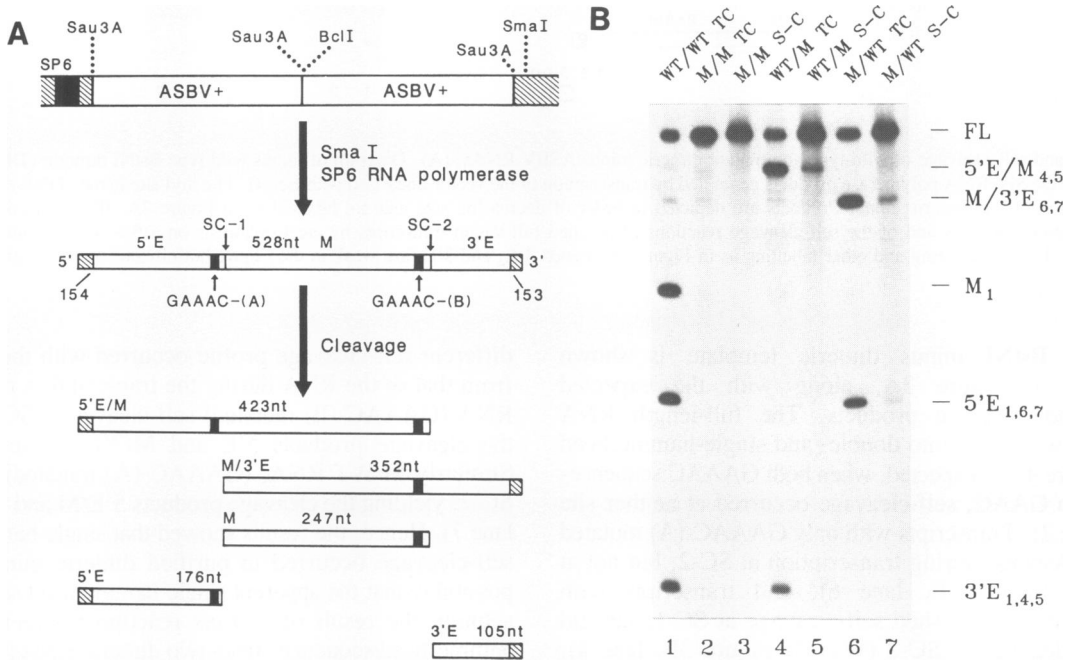

Figure 2. Synthesis and self-cleavage of wild-type and mutant dimeric plus ASBV RNAs. (A) Diagram of plus wild-type Sau3A dimeric cDNA clone of ASBV in pSP64 vector and the SP6 RNA polymerase products generated by transcription of the vector linearised with SmaI. Products are depicted in order of decreasing size. Self-cleavage at both sites (SC-1 and SC-2, arrowed) of the full-length transcript (FL) gave rise to a 5′-end fragment (5′E), a monomer fragment (M) and a 3′-end fragment (3′E). Also shown are the products when self-cleavage occurred only at SC-1 (5′E and M/3′E), or only at SC-2 (5′E/M and 3′E). Hatched boxes indicate vector sequences at 5′- and 3′-ends of both the cDNA clone and RNA transcripts; closed boxes, GAAAC sequences (Figure 1) labelled A and B and indicated by arrows; large black box, SP6 RNA polymerase promoter. Relevant restriction sites in the cDNA clone are indicated. ASBV sequence is numbered after Symons (35). (B) Analysis of the SP6 RNA polymerase transcription (TC) reactions and of the self-cleavage (S-C) reactions of purified products by electrophoresis on a 5% polyacrylamide, 7 M urea gel and autoradiography. The positions of the products are indicated on the right-hand side of the gel and correspond to those in (A); the subscript numbers refer to the lanes in which the bands occur. Lane 1; transcript of the wild-type Sau3A dimeric template linearised with SmaI (WT/WT TC). Lane 2; as for lane 1, but both template GAAAC sequences (A and B) mutated to GAAC (M/M TC). Lane 3; purified full-length M/M RNA (mutant at both GAAAC sequences) incubated under standard self-cleavage conditions (refer to Materials and Methods) (M/M S-C). Lane 4; as for lane 2 but only GAAAC-(B) mutated (WT/M TC). Lane 5; as for lane 3 but with purified full-length WT/M RNA (WT/M S-C). Lane 6; as for lane 2 but only GAAAC-(A) mutated (M/WT TC). Lane 7; as for lane 3 but with purified full-length M/WT RNA (M/WT S-C). Note that bands $5'E_7$ and $3'E_5$ are weak in the Figure but obvious on the original autoradiogram.

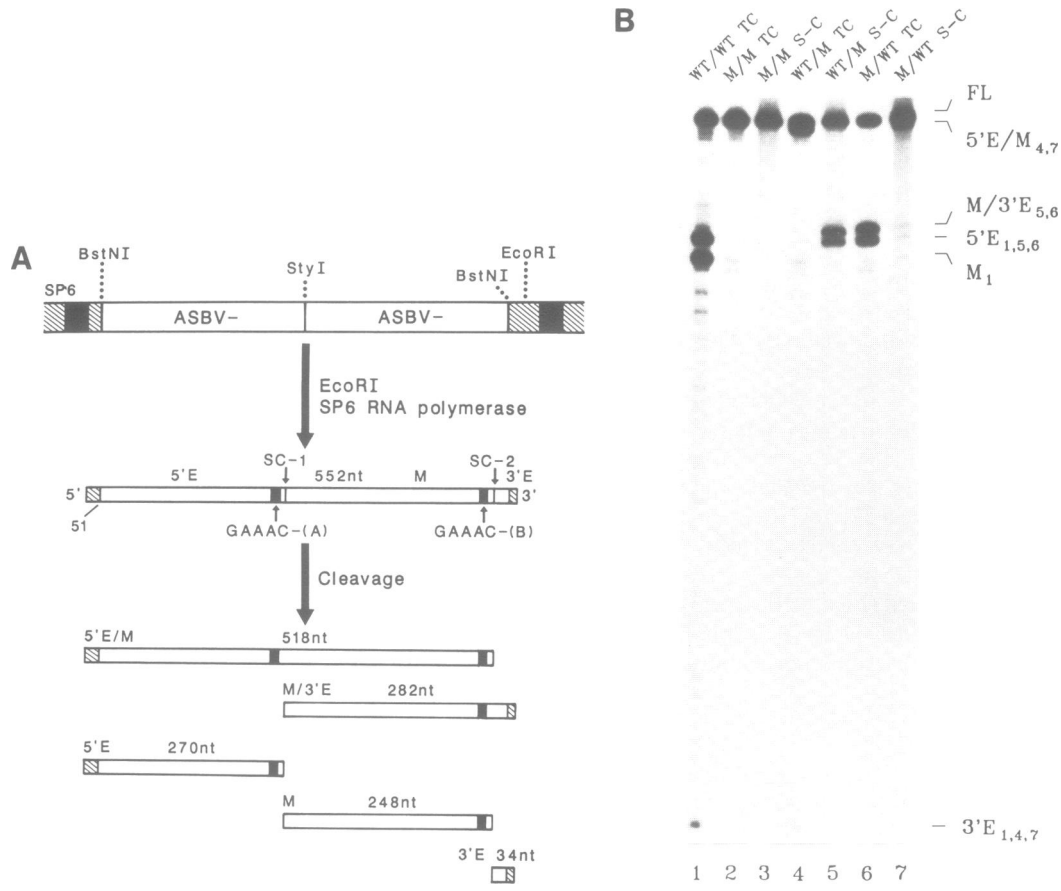

Figure 3. Synthesis and self-cleavage of wild-type and mutant dimeric minus ASBV RNAs. (A) Diagram of minus wild-type BstNI dimeric cDNA clone of ASBV in pGem1 vector and the SP6 RNA polymerase products generated by transcription of the vector linearised with EcoRI. The StyI site in the cDNA clone was generated during the construction of the dimeric clone. Products are depicted in order of decreasing size and are labelled as in Figure 2A. (B) Analysis of the SP6 RNA polymerase transcription reactions and of the self-cleavage reactions of purified full-length transcripts by electrophoresis on a 5% polyacrylamide, 7 M urea gel and autoradiography. Lane numbering and other labelling as in Figure 2B. Bands $3'E_4$ and $3'E_7$ are weak in the Figure but obvious on the original autoradiogram.

The pGem1 BstNI minus dimeric template is shown diagramatically in Figure 3A, along with the expected transcription and cleavage products. The full-length RNA transcript is shown folded into double- and single-hammerhead strutures in Figure 4. As expected, when both GAAAC sequences were mutated to GAAC, self-cleavage occurred at neither site (Figure 3B, lane 2). Transcripts with only GAAAC-(A) mutated abolished self-cleavage during transcription at SC-2, but not at SC-1 (M/WT, Figure 3B, lane 6), and transcripts with GAAAC-(B) mutated abolished self-cleavage at SC-1, but did not affect self-cleavage at SC-2 (WT/M; Figure 3B, lane 4), indicating that these RNAs self-cleaved by a double-hammerhead structure. Hence, during transcription, minus dimeric RNAs, like the plus dimeric RNAs, self-cleaved by a double-hammerhead structure.

Purified Dimeric Minus ASBV RNA Self-Cleaves By a Single-Hammerhead Structure

Purified full-length mutated dimeric minus ASBV RNA transcripts were subjected to standard self-cleavage conditions. Figure 3B, lanes 5 and 7, show the self-cleavage pattern for the WT/M RNA (GAAAC-(B) mutated) and the M/WT RNA (GAAAC-(A) mutated), respectively. As is clearly evident, a different self-cleavage profile occurred with the purified RNA from that of the RNA during the transcription reaction. WT/M RNA (GAAAC-(B) mutated) self-cleaved at SC-1, resulting in the cleavage products 5'E and M/3'E (Figure 3B, lane 5). Similarly M/WT RNA, (GAAAC-(A) mutated) self-cleaved at SC-2, yielding the cleavage products 5'E/M and 3'E (Figure 3B, lane 7). Hence, the results showed that single-hammerhead RNA self-cleavage occurred in purified dimeric minus RNAs. The possibility that the apparent single-hammerhead self-cleavage was actually the result of a *trans* reaction between two wildtype hammerhead sequences from two dimeric RNAs is very unlikely since the self-cleavage reactions of the isolated RNAs were conducted at very low concentrations of RNA (approximately 0.3 nM; 50 ng/ml).

In addition to the single-hammerhead self-cleavage, a small amount of double-hammerhead self-cleavage also occurred as indicated by the presence of trace amounts of 5'E/M and 3'E in the WT/M reaction (Figure 3B, lane 5) and of 5'E and M/3'E in the M/WT self-cleavage reaction (Figure 3B, lane 7).

We consider that conformational changes occurred during the post-transcriptional treatment of the purified RNA that allowed some of the RNA to fold into a single-hammerhead structure and a much smaller fraction into a double-hammerhead structure. On the other hand, during the transcription reaction the nascent RNA

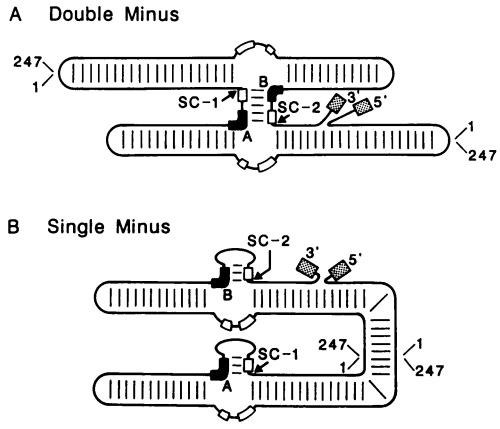

Figure 4. Schematic representation of a dimeric minus ASBV RNA transcribed from wild-type BstNI dimeric cDNA clone in pSP64 vector (Figure 3A) and folded to contain double- (A) and single- (B) hammerhead structures. Self-cleavage sites, labelled SC-1 and SC-2, are indicated by arrows; stippled boxes, vector sequences at 5′- and 3′-ends; closed boxes, GAAAC sequences (Figure 1) labelled A and B; open boxes, remaining conserved nucleotides (Figure 1). Base-pairing is represented by lines between RNA strands. Sequence numbered after Symons (35).

presumably folded preferentially into a double-hammerhead structure, precluding the formation of the single-hammerhead structure.

DISCUSSION

The aim of this paper was to investigate the structures involved in the self-cleavage of plus and minus ASBV dimeric RNAs, both during the transcription reaction, and also after purification of the RNAs had allowed refolding of the RNAs into alternative conformations. Determination of the single- or double-hammerhead route in the dimeric RNA transcripts was made feasible by the use of single base deletion mutants whereby conversion of the conserved GAAAC sequence in the hammerhead structure to GAAC completely eliminated self-cleavage at one of the two sites. Inhibition of self-cleavage at the site just 3′ to the mutated GAAAC demonstrated single-hammerhead self-cleavage, whereas inhibition of self-cleavage at the other self-cleavage site (over 250 bases from the mutated GAAAC sequence) indicated double-hammerhead mediated self-cleavage (Ref. 2; and Figures 1, 4).

Plus and minus dimeric transcripts differed in that, although both RNAs self-cleaved during the transcription reaction by a double-hammerhead structure (Ref. 2; and Figures 2B, 3B), only minus dimeric transcripts self-cleaved by single-hammerhead structures as a purified RNA (Figures 2B, 3B, Table 1). The ability of the minus dimeric ASBV RNA to form active single-hammerhead structures, compared with the inability of the plus dimeric RNA to do so is consistent with the greater theoretical stability of the minus single-hammerhead stem III, as it has an extra base-pair compared with the plus single-hammerhead stem III (Figure 1).

The structures that the minus dimeric RNA formed, either during transcription or after purification, were dependent on the conditions the RNA was subjected to prior to and during the self-cleavage reaction. Nascent RNA that was able to fold as it emerged from the polymerase during transcription (for example, see Ref. 30) folded into a different active structure (the double-hammerhead structure) from the full-length purified RNA (predominantly single-hammerhead structures) where the total RNA sequence was present during the folding of the RNA. During the self-cleavage reactions, in addition to the formation of either the single-hammerhead or double-hammerhead structures, the minus dimeric RNA was folded into other, inactive, structures as indicated by the RNA that did not undergo self-cleavage and so appeared as full-length RNA, when analysed by gel electrophoresis (Figures 2B, 3B). The ability for other hammerhead containing RNAs to form alternative inactive structures has been reported extensively (for example, Refs. 5,8,10,26,27,31–34).

Table 1. Summary of the type of hammerhead structure mediating self-cleavage, during transcription and after purification, of dimeric plus and minus ASBV RNAs. Double; double-hammerhead structure mediates self-cleavage, single; single-hammerhead structure.

Polarity of Dimeric RNA	Self-Cleavage Mechanism	
	During Transcription	After Purification
Plus	Double	Double
Minus	Double	Mostly Single

The self-cleavage of minus dimeric RNA by both single- and double-hammerhead structures is an important finding, as it demonstrates that a single species of RNA can undergo self-cleavage by two different (but related) structures. Sheldon and Symons (26) have previously demonstrated that small RNAs containing hammerhead sequences were capable of either single-hammerhead self-cleavage or a combination of single- and double-hammerhead self-cleavage, depending upon the conditions under which the self-cleavage reactions were conducted. In addition, Ruffner et al. (27) found that two short oligoribonucleotides based on the minus ASBV hammerhead sequence, although capable of double-hammerhead structure formation and self-cleavage, preferentially self-cleaved by a single-hammerhead reaction. This paper, however, provides the first demonstration of an RNA containing a full-length viroid sequence having the capacity for self-cleavage by two different structures.

ASBV is believed to be replicated by a rolling circle mechanism (13,14) involving self-cleavage of multimeric replicative intermediates to form monomeric RNAs (15). It seems likely that self-cleavage *in vivo* would more closely resemble the self-cleavage of dimeric RNAs during *in vitro* transcription than the *in vitro* self-cleavage of gel-purified dimeric RNAs. This is because sequential production and folding of the greater than unit-length RNAs occurs during transcription. As both dimeric plus and minus RNAs self-cleaved by double-hammerhead structures during *in vitro* transcription, it seems probable that self-cleavage *in vivo* occurs by these structures also.

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