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Self-cleavage of plus and minus RNA transcripts of avocado sunblotch viroid

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ABSTRACT

Self-cleavage of both plus and minus RNA transcripts of the 247-residue avocado sunblotch viroid (ASBV), prepared from tandem dimeric cDNA clones, occurs specifically at two sites in each transcript to give monomeric plus and minus species. The cleavage reaction occurs both during transcription and on incubation of purified transcripts at pH 8 and 37°C in the presence of magnesium ions to give a 3'-terminal 2',3'-cyclic phosphate and a 5'-terminal hydroxyl group.

Although the self-cleavage occurs at different sites in the ASBV molecule for the plus and minus species, very similar secondary structures with high sequence homology can be drawn at each site. The results are considered to provide further evidence that ASBV is replicated in vivo by a rolling circle mechanism involving non-enzymic cleavage of high molecular weight RNA precursors of ASBV.

INTRODUCTION

Viroids, an unusual class of pathogens restricted to higher plants, are infectious single-stranded, circular RNAs of between 246 and 375 nucleotides. Replication of viroids does not involve a DNA intermediate and presumably relies solely on host proteins since there is no evidence for viroid-coded polypeptides (1-3). A rolling circle mechanism of replication is considered to account for the presence of greater-than-unit-length viroid (plus) and viroid-complementary (minus) RNAs in infected plants (4-8). An essential feature of such a mechanism is the specific processing or cleavage of plus and minus high molecular weight intermediates to produce plus or minus linear viroid monomers which are then ligated to circular monomers. We describe here the specific, non-enzymic cleavage (self-cleavage) of both plus and minus in vitro-synthesized RNA transcripts of dimeric cDNA clones of the 247-nucleotide avocado sunblotch viroid (ASBV) (9). Such reactions are considered to be an essential part of the replication of ASBV in vivo.

Northern hybridization analysis of extracts of leaves from ASBV-infected avocado trees detected an oligomeric series of plus species up to decamers but

only monomer and dimer minus species (7,8). Most of the monomer plus and part of the monomer minus species were present as circular molecules. Since the greater-than-unit-length plus and minus species were present at low concentrations, we found it difficult to investigate the mechanism of the putative processing or cleavage of these RNAs. Hence, we turned to the phage SP6 RNA polymerase transcription system (10) which allowed us to readily prepare defined plus and minus RNA transcripts of cDNA clones of ASBV *in vitro*.

MATERIALS AND METHODS

Construction of cDNA clones of ASBV

Tandem dimeric clones of ASBV were constructed by ligating full-length ASBV monomeric DNA inserts, excised with Sau3A from clones constructed in the phage DNA vector M13mp93 (11), under conditions to produce dimers. After confirmation of sequence and orientation by dideoxy sequence analysis (12), the dimeric ASBV insert and small flanking regions of the M13mp93 clones were excised with EcoRI and HindIII and recloned into the vectors pSP64 (Fig. 1a) and pSP65 (Fig. 1b) cut with the same enzymes. The full-length ASBV monomeric M13mp93 DNA clones used here contained a C to T base change at position 213 of the published ASBV sequence (9). SP6 RNA polymerase transcription of the SmaI-linearized ASBV dimer clone in pSP64 (Fig. 1a) was expected to generate a 528 nucleotide complete transcript (C) consisting of the ASBV plus dimer (494 residues) flanked by 27 residues of pSP64/M13mp93 sequence at the 5'-terminus and 7 residues of M13 sequence at the 3'-terminus. Transcription of the HindIII-linearized ASBV dimer clone in pSP65 (Fig. 1b) was expected to generate a full-length 534 residue minus transcript (C) with 20 residues of pSP65/M13mp93 sequence at the 5'-terminus and 20 residues of M13mp93 sequence at the 3'-terminus of the ASBV minus dimer.

Transcription reactions with SP6 RNA polymerase

ASBV dimer clones in pSP64 and pSP65 (2 μg) were linearized with SmaI or HindIII, respectively, and transcribed with SP6 RNA polymerase (1.6 units; BRESA, Adelaide) in a reaction mixture containing, with or without 100 μCi of γ-^{32}P-GTP or α-^{32}P-GTP (BRESA, Adelaide), 0.5 mM GTP, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, and 0.1 μg/μl bovine serum albumin. The same transcription products were obtained when RNasin (0.5 U/μl; Promega Biotech) was included, or when bovine serum albumin was replaced by 2 mM spermidine. SP6 RNA polymerase transcription of non-linearized clones in the presence of α-^{32}P-GTP was as described for the linearized clones.

Detection of the transcription products was by autoradiography after electrophoretic separation on a 5% polyacrylamide gel containing 7 M urea.

Cyclization of monomeric transcription products by wheat germ RNA ligase

Plus and minus monomeric products (M, Fig. 2a, 2b), labelled by incorporation of α-^{32}P-GTP during transcription, were eluted from denaturing acrylamide gels and ethanol precipitated. They were then incubated for 1.0 hour with a partially purified preparation (1 µl) of wheat germ RNA ligase (13) in 9 µl of 20 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2mM DTT, 0.2 mM ATP, and the ligation products analysed by electrophoresis on a 4% polyacrylamide gel containing 7 M urea. Wheat germ RNA ligase was prepared to the DEAE-cellulose chromatography stage (13).

Self-cleavage of purified transcription products

Products of transcription reactions, either unlabelled or labelled by incorporation of α-^{32}P-GTP, were eluted from denaturing polyacrylamide gels, ethanol precipitated, and incubated in 40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, at 40°C for 1 hour. Reaction mixtures were analysed by electrophoresis on 4% polyacrylamide, 7 M urea gels.

RESULTS

Cleavage of RNA transcripts occurs during transcription

Dimeric cDNA clones of ASBV were prepared (Fig. 1) in the pSP64 and pSP65 plasmid vectors which, on transcription with phage SP6 RNA polymerase, were expected to give plus and minus dimeric transcripts, respectively, of ASBV. SP6 RNA polymerase transcription of the SmaI-linearized pSP64 plus clone was predicted to generate a complete transcript C of 528 nucleotides containing a plus ASBV dimer sequence (Fig. 1a). However, three major plus products, M, 5'E and 3'E, in addition to lesser amounts of the putative full-length dimer transcript C were observed when the reaction mixture was analysed on a denaturing polyacrylamide gel (Fig. 2a, Lane 2). Product M co-electrophoresed with a marker (ML, not shown) of purified ASBV linear monomer (247 nucleotides, ref. 9) and indicated that an ASBV linear monomer was specifically cleaved from the full-length dimer transcript C.

The identity of the products smaller than the complete transcripts was investigated by carrying out two other types of transcription reactions, one in which only the 5'-terminal nucleotide of the complete transcript was labelled by using γ-^{32}P-GTP as the sole labelled nucleotide, and the other where the vector was not linearized, thus permitting transcription to continue well beyond the ASBV sequence into the vector sequence. Incorporation of

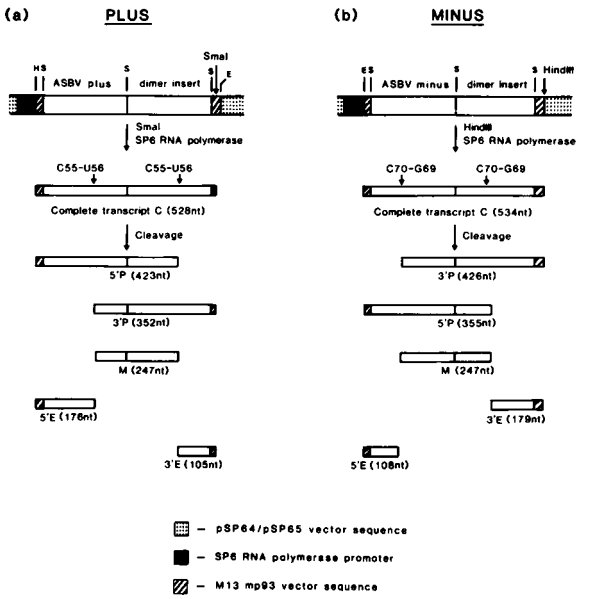

Figure 1. Diagram of plus and minus tandem cDNA clones of ASBV in pSP64 and pSP65 plasmid vectors and the SP6 RNA polymerase products generated by transcription of the vector linearized with SmaI (plus) and HindIII (minus). Products are depicted in order of decreasing size. The positions of self-cleavage in the complete transcripts C are indicated by short arrows. Partially cleaved fragments are labelled P, monomeric fragments, M, and end-fragments, E.

radioactive label into product 5'E and the residual complete dimer transcript C, but not into products M and 3'E, during transcription of the linearized vector in the presence of γ-^{32}P-ATP (Fig. 2a, Lane 1) established that products M and 3'E (Fig. 2a, Lane 2) were not the result of premature termination of transcription. When non-linearized vector was transcribed using α-^{32}P-GTP, only products M and 5'E plus a smear of high molecular weight material were observed; complete transcript C and 3'E were absent (Fig. 2a, Lane 3). It was concluded that product 5'E was the 5'-terminal fragment and product 3'E the 3'-terminal fragment resulting from the excision of product M, an ASBV linear monomer, from the complete transcript C (Fig. 1a). The minor amounts of products 5'P and 3'P (Fig. 2a, Lane 2) were considered to arise by cleavage at only one of the two possible cleavage sites in transcript C (Fig. 1a).

Estimates of the size of all fragments were obtained on separate 5% polyacrylamide, 7 M urea gels, by reference to single-stranded marker DNAs

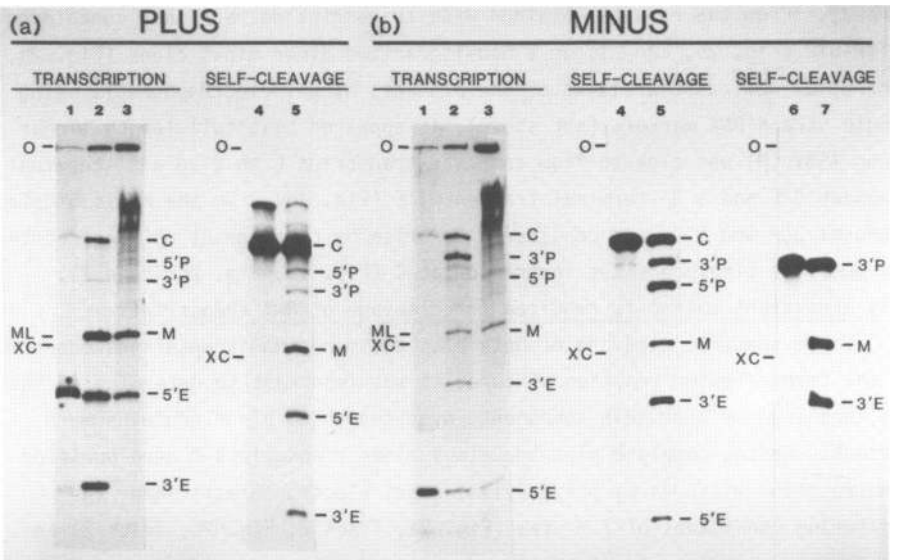

Figure 2. SP6 RNA polymerase transcription of dimeric cDNA clones of ASBV and self-cleavage reaction of purified products. Detection of transcription products was by autoradiography after separation by electrophoresis on 5% acrylamide, 7 M urea gels. The positions of products are indicated on the right hand side of each gel and correspond to the diagram of Fig. 1. The positions of the marker linear monomer ASBV (ML), detected by staining with 0.02% toluidine blue, and the xylene cyanol FF dye (XC) are indicated on the left hand side. O, origin of gel. In (a) and (b), Tracks 1 and 2 are transcription reactions with linearized vector, and Track 3 with non-linearized vector. γ-^{32}P-GTP was the only labelled nucleotide in the reactions of Track 1, and α-^{32}P-GTP the only labelled nucleotide in the reactions of Tracks 2 and 3. The two tracks 4 contain the non-incubated complete transcript C, isolated from the corresponding Track 2; no self-cleavage occurred when each complete transcript C was incubated in 40 mM Tris-HCl, pH 8.0, 1 mM EDTA, at 40°C for 1 hour (not shown). Track 6 contains the non-incubated minus product 3'P isolated from the corresponding Track 2. The two Tracks 5 and Track 7 contain the products of the self-cleavage reaction in 40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, at 40°C for 1 hour.

obtained on denaturation of the plasmid vector pUC19 after digestion with HpaII (BRESA, Adelaide) (data not given). All these estimates were found to have an error of only 1 - 5% when the precise cleavage sites and fragment sizes were subsequently determined.

Similar results were obtained on the SP6 RNA polymerase transcription of the HindIII-linearized pSP65 ASBV minus dimer clone (Fig. 1b; Fig. 2b). Four major minus products, 3'P, M, 3'E and 5'E were obtained in addition to the putative full-length 534-nucleotide minus dimer transcript C (Fig. 1b; Fig. 2b

Lane 2). From the results obtained with transcription reactions containing γ-^{32}P-GTP (Fig. 2b, Lane 1) or a non-linearized dimer minus clone (Fig. 2b, Lane 3) as well as the sizing of the products by gel electrophoresis using single-strand DNA markers (not shown), it appeared that full-length linear minus ASBV (M) was cleaved from complete transcript C to give a 5'-terminal fragment 5'E and a 3'-terminal fragment 3'E (Fig. 1b). On the basis of size, products 3'P and 5'P were considered to arise by cleavage at only one of the two possible cleavage sites in transcript C (Fig. 1b, Fig. 2b, Lane 2).

Only a divalent cation is required for cleavage of RNA transcripts

Since specific cleavage of both plus and minus RNA transcripts occurred in the transcription reaction mixture, it was important to determine if this was catalysed by a protein component, even though no plant proteins were present. Hence, complete plus and minus dimer transcripts C were purified from reaction mixtures by polyacrylamide gel electrophoresis under the denaturing conditions of 7 M urea (Fig. 2a, Track 2; Fig. 2b, Track 2) and tested for specific self-cleavage under a wide range of conditions. The only component consistently required for self-cleavage, in addition to the RNA, was a divalent metal ion, either Mg^{2+} or Ca^{2+}. For example, incubation of transcripts at 40°C in 40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, for one hour showed significant self-cleavage of both plus (Fig. 2a, Lane 5) and minus (Fig. 2b, Lane 5) transcripts. The presence of products 5'P and 3'P indicated cleavage at only one of the two possible sites (Fig. 1). That only one complete site is necessary for self-cleavage was shown by the incubation of the minus product 3'P (Fig. 2b, Track 2) under the same conditions to give products M and 3'E (Fig. 2b, Tracks 6 and 7). The extent of self-cleavage has varied widely in numerous reactions carried out in the past year. For example, after one hour in a higher concentration of Mg^{2+} (50 mM) at 40°C, self-cleavage of the minus transcript C was essentially complete. The purified minus transcript C always self-cleaved more efficiently than the plus transcript C (data not shown). A comprehensive study of the effect of the concentration of different cations on the rate and extent of self-cleavage has yet to be done.

Self-cleavage of plus and minus transcripts occurs at two different sites in the ASBV molecule

The sites of cleavage of the plus and minus transcripts were determined by direct enzymic sequencing (14-17) of 5'-^{32}P-labelled products. Plus products M and 3'E, purified from a non-radioactive transcription reaction mixture (Fig. 2a, Track 2), and minus product 3'E, purified from a self-cleavage reaction of non-radioactive 3'P (Fig. 2b, Track 7), could be 5'-^{32}P-

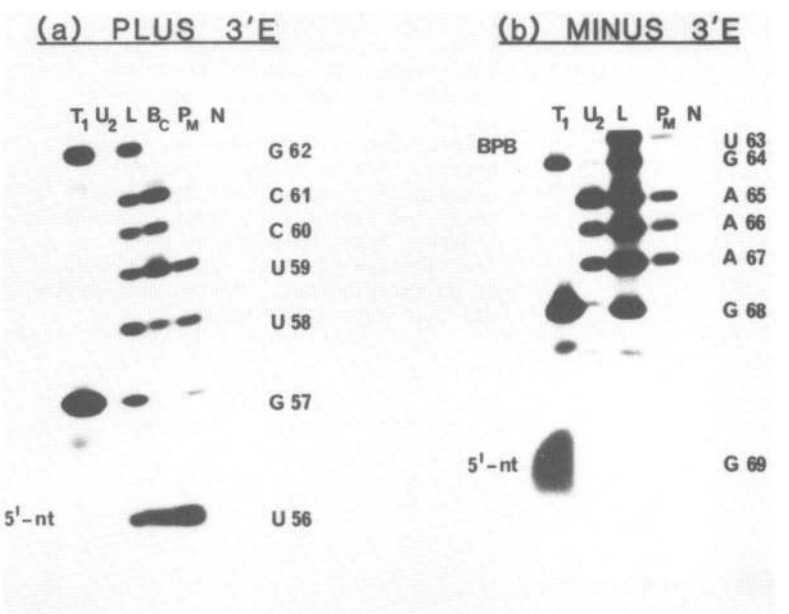

Figure 3. Enzymic sequencing of plus product 3'E (a), purified from a transcription reaction mixture, and minus product 3'E (b), purified after a self-cleavage reaction of 3'P. Non-radioactive products used for sequencing were detected on polyacrylamide gels by staining with 0.02% toluidine blue and eluted. Enzymic sequencing after 5'-terminal labelling with γ-^{32}P-ATP was as described (14). Reactions were fractionated by electrophoresis on 20% polyacrylamide, 7 M urea gels and autoradiographed. Only the first seven residues are shown. T_1, T_1 RNase; U_2, U_2 RNase; Bc, Bacillus cereus RNase; Pm, Physarum polycephalum RNase; L, ladder generated by heating labelled product in formamide containing 2 mM $MgCl_2$ at 100°C for 5 min; N, no enzyme treatment. The position of the 5'-nucleotide (5'-nt) is indicated as well as each residue and residue number in the sequence of ASBV (9). The terminal 5'-nt in the ladder (L) of minus 3'E, not visible here, was present on the original autoradiogram.

labelled with T4 polynucleotide kinase and γ-^{32}P-ATP at pH 9.0 without prior dephosphorylation, indicating the presence of a free 5'-hydroxyl (17). Direct enzymic sequencing of the RNAs showed that the cleavage reaction had occurred between residues C55 and U56 of the plus species and between residues C70 and G69 of the minus species (Fig. 3a, b; sequence of plus M not shown). (In the complementary sequence, the same residue numbers as the plus viroid are retained; therefore, the minus strand is numbered in the 3'- to 5'-direction).

Analysis of the plus and minus monomeric products M indicated that they terminated in a 2',3'-cyclic phosphate (C>p). Firstly, ligation of α-^{32}P-GTP-labelled plus and minus products M (Fig. 4, Tracks 1 and 3) with a partially

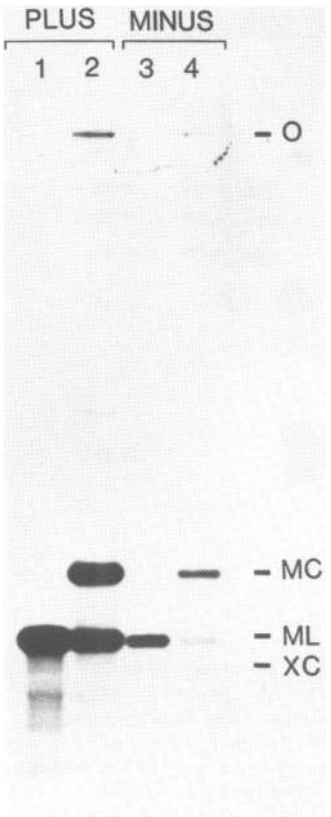

Figure 4. Cyclization of plus and minus ^{32}P- products M with wheat germ RNA ligase. MC and ML, positions of monomer circular and monomer linear markers (7) detected by staining. XC, xylene cyanol FF marker dye. O, gel origin. Tracks 1 and 3, control products M; Tracks 2 and 4, products M after incubation with wheat germ RNA ligase. Reaction mixtures were fractionated by electrophoresis on a 5% polyacrylamide, 7 M urea gel, which was then autoradiographed.

purified wheat germ RNA ligase preparation (13) gave major products which co-electrophoresed on a denaturing polyacrylamide gel with purified circular ASBV monomer (Fig. 4, Tracks 2 and 4). Wheat germ RNA ligase specifically requires a 2',3'-cyclic phosphate terminus for ligation with either a 5'-hydroxyl or a 5'-phosphorylated terminus (13,19). Secondly, plus product M could not be 3'-^{32}P-labelled with 5'-^{32}P-pCp using T4 RNA ligase, even after extensive treatment with alkaline phosphatase (18); this was not due to cyclization of plus product M by the T4 RNA ligase, as determined by denaturing gel electrophoresis and staining (data not shown). Finally, a minor spot, in addition to 5'-^{32}P-CMP, was observed after cellulose thin layer chromatography in two solvent systems (17) of nuclease P1-digestion plus product M labelled by the incorporation of α-^{32}P-CTP during transcription. This spot had the same R_f as 5'-^{32}P-pC>p prepared from RNase A-digested 5'-^{32}P-labelled poly(C) (data not shown). The presence of a 3'-terminal C supported the assignment of a unique cleavage site between C55 and U56 (Fig. 3a).

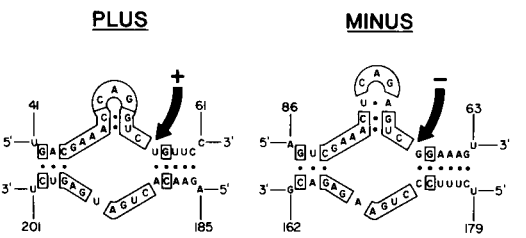

Figure 5. Secondary structures proposed around the plus and minus self-cleavage sites of ASBV. Residues conserved between the plus and minus structures are boxed and are numbered according to the proposed native structure of ASBV (9). The site of self-cleavage is indicated by an arrow.

In summary, the data indicate that RNA transcripts containing dimeric plus or minus ASBV sequences undergo self-cleavage at two specific phosphodiester linkages in each transcript to generate monomeric plus or minus ASBV sequences with 5'-hydroxyl and 2',3'-cyclic phosphate termini.

Proposed secondary structures at the processing sites of plus and minus ASBV are very similar

Proposed secondary structures around the self-cleavage sites of plus and minus ASBV are given in Fig. 5. The salient feature of these structures is the identical arrangement of 23 residues (boxed) conserved between the plus and minus cleavage sites (arrowed) around a central interior loop formed by the junction of three base-paired stems. Although the uppermost stem in the plus and minus structures appears to be thermodynamically unlikely, it may form transiently or it may be stabilized by divalent metal ions or by the formation of non-Watson-Crick base pairs (21), such as A:G base pairs (22, 23), and tertiary interactions such as the base triplets that occur in the three-dimensional structure of tRNA (21). In the plus strand, this structure may be formed by only a minor rearrangement of the secondary structures originally proposed for ASBV (9, 20).

The two-dimensional structures obviously do not explain why there is specific non-enzymic cleavage of plus and minus transcripts of ASBV. We propose that, in the presence of a divalent cation such as Mg^{2+} or Ca^{2+}, these structures (Fig. 5) form active tertiary complexes that lower the activation energy sufficiently at the specific internucleotide bond of the cleavage site to allow non-hydrolytic cleavage that results in the characteristic 5'-hydroxyl and 2',3'-cyclic phosphate termini. Further consideration of the functional significance of these proposed structures is in the Discussion.

The predicted cleavage structures of ASBV (Fig. 5) come from separate

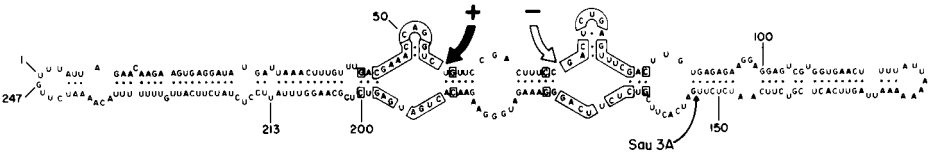

Figure 6. Proposed native structure of ASBV (9) modified to incorporate the plus self-cleavage structure and the complement of the minus self-cleavage structure (Fig. 5). The Sau3A site used for the construction of cDNA clones is indicated.

regions of the ASBV molecule (Fig. 6). On the basis of these structures, the ASBV molecule may be assigned two functional domains, one responsible for the cleavage of plus sequences and the other for minus sequences, that together account for approximately 30% of the ASBV sequence (Fig. 6). A detailed discussion of structural and functional domains in other viroids has been published (24).

DISCUSSION

We have described the specific self-cleavage of both plus and minus RNA transcripts of ASBV. The mechanism by which ASBV plus and minus RNAs cleave appears different from that of other reported forms of RNA self-cleavage. Cleavage of ASBV dimeric transcripts gives 5'-hydroxyl and 2',3'-cyclic phosphate termini and requires a divalent cation. Autoexcision of the intervening sequences from precursors of *Tetrahymena* ribosomal RNA (25-27), *Neurospora* cytochrome *b* mRNA (28) and several yeast mitochondrial RNAs (29) requires the presence of guanosine or GTP and results in the generation of 5'-phosphate and 3'-hydroxyl termini prior to cyclization of the excised sequence. The M1 RNA component of *Escherichia coli* RNase P catalyses the cleavage of tRNA precursors to produce 5'-phosphate and 3'-hydroxyl termini (30). Although self-cleavage of the phage T4 precursor RNA p2Spl gives 5'-hydroxyl and 3'-phosphorylated termini, divalent cations are not an absolute requirement for self-cleavage (31). In addition, no signficant sequence homology around the cleavage sites of any of these RNAs could be found with the conserved sequence of ASBV (Fig. 5).

The self-cleavage of a plus RNA transcript of a dimeric cDNA clone of potato spindle tuber viroid (PSTV) has been reported but the level of cleavage was low (1 to 5%) and the site of cleavage was identified as being in a 20 residue region (32). Infectivity and *in vitro* mutagenesis studies on cDNA clones of the related citrus exocortis viroid (CEV) have indicated that

cleavage in vivo of RNA precursors occurs at one of three adjacent residues in the central conserved region found in all viroids except ASBV (33) but opposite the region of cleavage identified for PSTV (32). Equivalent secondary structures to those proposed for the self-cleavage of plus and minus ASBV (Fig. 5) have not been found in the regions believed to be involved in the cleavage of precursor RNAs of these two viroids.

Although several different secondary structures are feasible at the plus and minus cleavage sites, an indication that the secondary structures in Fig. 5 may be of functional significance is that similar secondary structures with high sequence homology may be drawn around the putative or demonstrated processing sites of other plant pathogenic RNAs (unpublished data; reviewed in ref. 36). The structures are feasible at the putative processing sites of the greater-than-unit-length RNAs of the circular satellite RNAs or virusoids associated with solanum nodiflorum mottle virus (SNMV; ref. 8,14,37), velvet tobacco mottle virus (VTMoV; ref. 8,14,37), and subterraneum clover mottle virus (SCMoV; ref. 38,39) as well as around the demonstrated self-cleavage site of dimers of the linear satellite RNA of tobacco ringspot virus (TRSV; ref. 40,41), and the self-cleavage sites of the plus and minus RNAs of the virusoid of lucerne transient streak virus (LSTV; ref. 8,42, unpublished).

Evidence for processing at these sites in vivo has been established for the closely related SNMV and VTMoV virusoids (378 and 366 residues, respectively; ref. 14) with the demonstration of a 2'-phosphomonoester, 3',5'-phosphodiester linkage between residues C49 and A50 (37). This linkage was considered (37) to be legacy of the in vivo cyclization of linear monomers with plant RNA ligases (19). The putative processing sites of the SNMV, VTMoV and SCMoV virusoids were originally proposed on the basis of their sequence homology with the 3'- and 5'-terminal regions of the linear satellite RNA of TRSV (P. Keese, personal communication, refs. 39,43). This linear satellite RNA is produced by self-cleavage of natural linear plus dimers (40).

The observation that both plus and minus sequences of ASBV self-cleave is further evidence for a rolling circle model for the replication (4-8) of ASBV, with the ASBV plus and minus monomers arising from processing rather than specific initiation and termination of transcription. The efficient cleavage of plus and minus ASBV dimers during transcription in the SP6 RNA polymerase system suggests that self-cleavage to produce linear monomers may occur during viroid transcription in vivo when sufficient sequence to enable the formation of a cleavage structure has been transcribed. The resultant plus and minus ASBV monomers would possess 5'-hydroxyl and 2'-3'-cyclic phosphate termini

suitable for either intermolecular ligation to produce oligomers or intramolecular ligation to produce circular ASBV plus and minus monomers with an enzyme of similar specificity to wheat germ RNA ligase (19). This is consistent with the ability to circularize at least a fraction of the linear viroids isolated from plants infected with potato spindle tuber viroid (34,35) and ASBV (unpublished data) with the wheat germ RNA ligase. The self-cleavage of both plus and minus ASBV sequences correlates with the detection of circular and linear monomer, as well as integral multimeric forms of plus and minus ASBV sequences, in extracts from ASBV-infected avocado trees by Northern hybridization analysis (7, 8).

An important aspect of the self-cleavage of in vitro-synthesized dimeric plus and minus RNAs of ASBV is that it allows the production in vitro of purer and larger amounts of plus and minus ASBV monomers than can be readily obtained from infected plants. These monomers will be useful for structural and infectivity studies.

Of the nine viroids that have been sequenced, eight share appreciable structural and sequence homology (24). ASBV is the exception (9), indicating that it has evolved from a different ancestor. The presence of significant sequence and structural homology around the demonstrated or putative cleavage sites of ASBV, the four virusoids and the satellite RNA of TRSV (unpublished) suggests that all these RNAs are evolutionarily related. It will be of considerable interest to characterize the replication and processing mechanisms of non-ASBV viroids and to compare them with those of ASBV, the virusoids and the satellite RNA of TRSV.

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