Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 12813–12817, December 1994
Biochemistry

Replication of avocado sunblotch viroid: Evidence for a symmetric pathway with two rolling circles and hammerhead ribozyme processing

(RNA self-cleavage/satellite RNAs)

JOSÉ A. DARÒS, JOSÉ F. MARCOS, CARMEN HERNÁNDEZ, AND RICARDO FLORES*

Unidad de Biología Molecular y Celular de Plantas, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Calle Jaime Roig 11, 46010 Valencia, Spain

Communicated by George Bruening, August 4, 1994

ABSTRACT **The structure of a series of RNAs extracted from avocado infected by the 247-nt avocado sunblotch viroid (ASBVd) was investigated. The identification of multistranded complexes containing circular ASBVd RNAs of (+) and (−) polarity suggests that replication of ASBVd proceeds through a symmetric pathway with two rolling circles where these two circular RNAs are the templates. This is in contrast to the replication of potato spindle tuber viroid and probably of most of its related viroids, which proceeds via an asymmetric pathway where circular (+)-strand and linear multimeric (−)-strand RNAs are the two templates. Linear (+) and (−) ASBVd RNAs of subgenomic length (137 nt and about 148 nt, respectively) and one linear (+)-strand ASBVd RNA of supragenomic length (383–384 nt) were also found in viroid-infected tissue. The two linear (+)-strand RNAs have the same 5′- and 3′-terminal sequences, with the supragenomic species being a fusion product of the monomeric and subgenomic (+)-strand ASBVd RNAs. The 3′ termini of these two (+)-strand molecules, which at least in the subgenomic RNA has an extra nontemplate cytidylate residue, could represent sites of either premature termination of the (+)-strands or specific initiation of the (−)-strands. The 5′ termini of sub- and supragenomic (+)-strand and the 5′ terminus of the subgenomic (−)-strand ASBVd RNA are identical to those produced in the *in vitro* self-cleavage reactions of (+) and (−) dimeric ASBVd RNAs, respectively. These observations strongly suggest that the hammerhead structures which mediate the *in vitro* self-cleavage reactions are also operative *in vivo*.**

Viroids are independently replicating circular RNAs capable of causing disease in infected plants. Replication of viroids has been proposed to occur through a rolling circle mechanism (1), because of the detection of multimeric negative (−)-strand RNAs [complementary to the predominant infectious (+)-strand] in tomato infected by the potato spindle tuber viroid. Other authors have come to similar conclusions (2, 3) and have also identified multimeric (+)-strand viroid RNAs in avocado infected by avocado sunblotch viroid (ASBVd) (4, 5) and in other viroid–host combinations (5–8). To accommodate these observations, two pathways were offered (ref. 6; see ref. 9 for a review). In the first pathway, the circular (+)-strand viroid RNA is transcribed into linear multimeric (−)-strands, which then act as the template for the generation of the linear multimeric (+)-strands; this pathway is termed asymmetric since it has a single rolling circle. In the second pathway, the linear multimeric (−)-strands are processed and ligated to monomeric circular (−)-strand RNA, which serves as the template for synthesis of the linear multimeric (+)-strand RNAs. This is the symmetric pathway, with two rolling circles. In both cases the multimeric (+)-strands resulting from the second round of RNA–RNA transcription are cleaved to unit-length molecules which are then ligated to the mature circular (+)-strand RNA. Evidence has been reported for a single rolling circle in the replication of potato spindle tuber viroid (10). However, the finding that dimeric ASBVd RNAs of both polarities self-cleave *in vitro* (11), a property also shared by the RNAs of peach latent mosaic viroid (12), provides indirect evidence against either polarity of multimeric RNA serving as a template for these two viroids, as well as for some satellite RNAs which display *in vitro* self-cleavage of their linear multimeric (+)- and (−)-strands (13, 14). The search for the presence in ASBVd-infected tissue of the circular monomeric (−)-strand, the key feature of the symmetric pathway, has not provided definitive results (4, 5), due to the difficulty in detecting this species by Northern analysis in the presence of a large excess of the circular monomeric (+)-RNA (5, 6, 9). Moreover, template circular (−)-strands should be isolated in complexes with (+)-strands (10); these complexes, however, would have remained unnoticed under the denaturing conditions used (4, 5).

A second group of questions pertains to whether viroid strands have specific sequences for the initiation and/or termination of transcription, as well as for processing of oligomeric intermediates. For ASBVd, the sequences of the hammerhead structures mediating the *in vitro* self-cleavage (11) could also operate *in vivo*. One way to address these questions is to characterize defined replicative intermediates, but this approach has been hampered by the low levels to which they accumulate in most cases. However, a series of viroid (+)-RNAs not multiples of the unit length have been identified in ASBVd-infected tissue as possible intermediates of the replication cycle (4, 5). Here we report the characterization of some of them, together with the detection of the monomeric circular (−)-RNA forming part of multistranded complexes. These results provide direct evidence for ASBVd replication proceeding through a symmetric pathway with hammerhead ribozymes being involved in processing of the linear multimeric RNAs.

MATERIALS AND METHODS

Nucleic acids from healthy or ASBVd (Spanish isolate)-infected (15) avocado plants (*Persea americana* Miller) were

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Abbreviations: ASBVd, avocado sunblotch viroid; *mc* and *ml*, monomeric circular and linear; *dc* and *dl*, dimeric circular and linear; *sub* and *sup*, subgenomic and supragenomic; STE, 100 mM NaCl/50 mM Tris·HCl, pH 7.2/1 mM EDTA.

*To whom reprint requests should be addressed at the present address: Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Universidad Politécnica de Valencia, Camino de Vera 14, 46022 Valencia, Spain.

extracted with buffer-saturated phenol and fractionated on nonionic cellulose (CF11; Whatman) with STE (100 mM NaCl/50 mM Tris·HCl, pH 7.2/1 mM EDTA) containing 35% ethanol (16) or, when indicated, 25% ethanol. Polysaccharides were removed with methoxyethanol (17).

RNAs were examined in nondenaturing 5% polyacrylamide gels with TAE (40 mM Tris/20 mM sodium acetate/1 mM EDTA, pH 7.2) (18) and/or urea/5% polyacrylamide gels with TBE (89 mM Tris/89 mM boric acid/2.5 mM EDTA, pH 8.3) for the electrode buffer and TBE plus 8 M urea for the gel buffer (19). Urea gels of smaller pore size (10% polyacrylamide) or lower ionic strength (0.25× TBE) (20), were also used. After ethidium bromide staining the RNAs were either eluted or electroblotted to nylon membranes and fixed by UV irradiation. Full-length ASBVd transcripts of both polarities, labeled with ^{32}P, were produced from two plasmids containing an ASBVd insert in either orientation (21). After prehybridization, hybridization (at 70°C in the presence of 50% formamide), and washing [at 55°C in 0.1× SSC (15 mM NaCl/1.5 mM sodium citrate, pH 7.0/0.1% SDS) (22), the membranes were autoradiographed.

Purified subgenomic ASBVd (+)-strand RNA was polyadenylylated (23). First-strand cDNA was synthesized by using $p(dT)_{15}$ primer and avian myeloblastosis virus reverse transcriptase (24), and the second strand by using RNase H and DNA polymerase I (25). The double-stranded cDNA was treated with T4 DNA polymerase, cloned in the *Sma* I site of plasmid pUC18, and sequenced (26). The sequence of a portion of the 5′ region of this RNA was determined by primer extension using chain-terminating inhibitors (27) and primer I complementary to nt 94–114 of ASBVd. The structure of supragenomic ASBVd (+)-strand [*sup*-ASBVd(+)] RNA was inferred from analyses by urea/1× TBE PAGE of the cDNAs obtained by primer extensions (28) using either primers II or III, complementary to nt 42–57 and 157–191 of ASBVd, respectively. In some cases reaction mixtures contained chain-terminating inhibitors. Plus and minus monomeric circular (*mc*) ASBVd (+) and (−) RNAs were identified by reverse transcription using respectively primers III (see above) and IV (homologous to ASBVd nt 70–98).

RESULTS

ASBVd RNAs of Genomic, Subgenomic, and Supragenomic Length Are Present in ASBVd-Infected Tissue. Fig. 1 shows the ASBVd RNAs separated in urea gels and detected by hybridization with ASBVd probes. *mc*- and *ml*-ASBVd(+), previously characterized by sequencing (15) and primer extension (28), respectively, were the most abundant species. *dc*- and *dl*-ASBVd(+) RNAs were also present at a high level. That these bands were actually the dimeric forms was inferred from their mobilities in urea gels. Moreover, *dc*- and *mc*-ASBVd(+) RNAs generated the same oligonucleotide patterns after limited digestions with RNase T1 or U2, and among the degradation products of *dc*-ASBVd were *dl* and *ml*-ASBVd (data not shown). The weaker signals observed with the probe for ASBVd (−)-strand RNAs (Fig. 1*B*) could be interpreted by assuming that *mc*- and *dc*-ASBVd(−) RNAs actually exist and/or that they resulted from imperfect hybridization of the great excess of *mc*- and *dc*-ASBVd(+) RNAs with the probe of the same polarity. The band migrating between *mc*- and *dl*-ASBVd RNAs must be produced by an ASBVd (+)-strand RNA, since it hybridized preferentially with the probe for ASBVd (+)-strand RNAs. This RNA was termed *sup*-ASBVd(+), since its mobility (Fig. 1*A*, lane 1) suggested a size of about 400 nt.

Two other bands were observed in the lower part of the gels (Fig. 1, lanes 1). The band with the highest electrophoretic mobility showed strong and weak signals in the blots hybridized with the probes for ASBVd (+)- and (−)-strand RNAs,

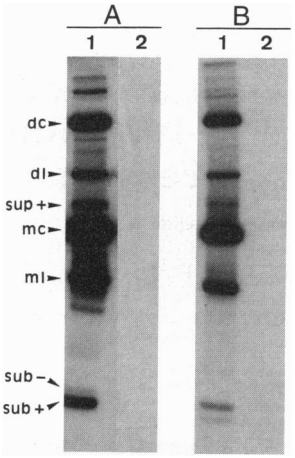

FIG. 1. Northern blot hybridizations of avocado nucleic acids after urea/5% PAGE, using RNA probes for (+) (*A*) and (−) (*B*) ASBVd sequences. Before loading, the samples dissolved in the gel buffer were denatured at 100°C for 1.5 min and quickly cooled on ice. Lanes 1 and 2, nucleic acids from ASBVd-infected and uninfected leaves, respectively. Arrowheads indicate dimeric circular (*dc*) and linear (*dl*) (+)-strand, monomeric circular (*mc*) and linear (*ml*) (+)-strand, supragenomic (+)-strand (*sup*+), and subgenomic (+)-strand (*sub*+) and (−)-strand (*sub*−) ASBVd RNAs. The acid-precipitable counts of the probe in *B* were 3-fold those in *A*. The autoradiograms were overexposed to make visible the *sub*+ and *sub*− ASBVd RNAs.

respectively. Therefore, the RNA producing this band was termed *sub*-ASBVd(+), and it is a linear RNA of 137 nt (see below). The band with the second highest electrophoretic mobility displayed a more intense signal in the blot hybridized with the probe for ASBVd (−)-strand RNAs. The corresponding RNA was termed *sub*-ASBVd(−), and it has an apparent size slightly greater than that of *sub*-ASBVd(+). Other bands above the *dc*-ASBVd RNA probably correspond to the higher-order multimers described previously (4, 5), which are not well resolved with the system used here.

Characterization of *sub*-ASBVd(+) and -(−). Attempts to observe with ethidium bromide the *sub*-ASBVd(+) RNA in 5% polyacrylamide gels with urea failed, probably because these gels do not resolve small RNAs. However, when 10% gels with urea were used, a differential band with the mobility of *sub*-ASBVd(+) was seen (data not shown). This RNA was eluted and its identity with *sub*-ASBVd(+) was confirmed by 5% PAGE and Northern blot analysis. Most of the *sub*-ASBVd(+) sequence was derived from cDNA clones obtained by polyadenylylating its 3′ end and priming synthesis of first-strand cDNA with $p(dT)_{15}$. The 18 nt proximal to the 5′ end were determined by reverse transcription with primer I and chain-terminating inhibitors. *sub*-ASBVd(+) is a linear RNA molecule of 137 nt identical to ASBVd nt 56–191, with an additional cytidylate residue at its 3′ end. The absence of this extra cytidylate residue in *mc*-ASBVd(+) (15) was confirmed by direct sequencing (data not shown). Both 3′ and 5′ termini of *sub*-ASBVd(+) have free hydroxyl groups, since it could be polyadenylylated (see above) and phosphorylated by polynucleotide kinase and [γ-^{32}P]ATP (data not shown). Therefore, the 5′ end of *sub*-ASBVd(+) is coincident with that produced by the *in vitro* self-cleavage of dimeric ASBVd (+)-strand RNA (11).

Although the minimal amounts at which *sub*-ASBVd(−) accumulates have precluded a detailed characterization, some information about its structure was obtained. If it is assumed that the 5′ end of *sub*-ASBVd(−) results from the action of the (−)-strand self-cleavage domain, it should be located at nt 69 (Fig. 2*A*). Also, by analogy with the *sub*-ASBVd(+) RNA, which has its 3′ end at nt 191, correspond-

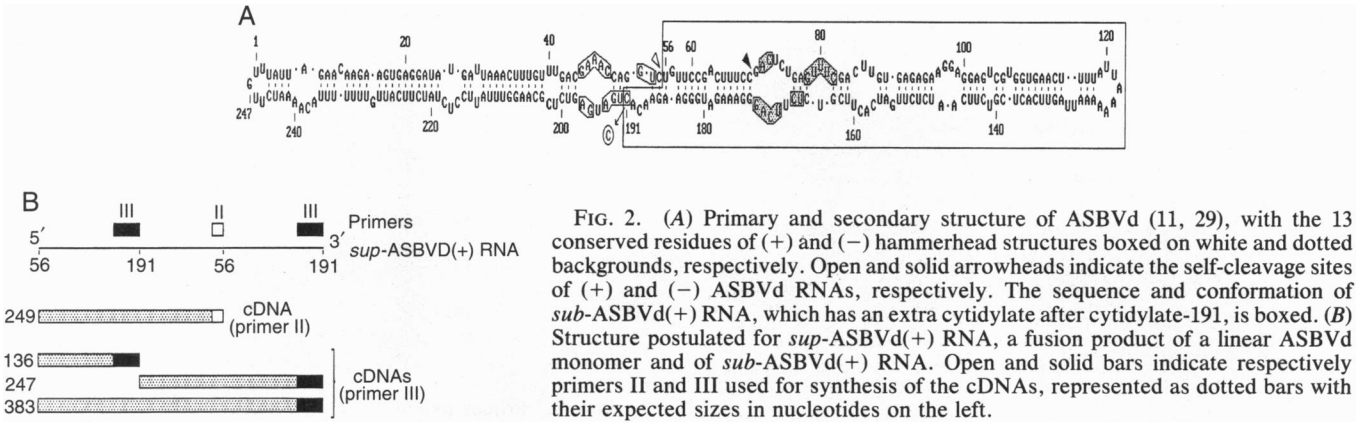

FIG. 2. (A) Primary and secondary structure of ASBVd (11, 29), with the 13 conserved residues of (+) and (−) hammerhead structures boxed on white and dotted backgrounds, respectively. Open and solid arrowheads indicate the self-cleavage sites of (+) and (−) ASBVd RNAs, respectively. The sequence and conformation of *sub*-ASBVd(+) RNA, which has an extra cytidylate after cytidylate-191, is boxed. (B) Structure postulated for *sup*-ASBVd(+) RNA, a fusion product of a linear ASBVd monomer and of *sub*-ASBVd(+) RNA. Open and solid bars indicate respectively primers II and III used for synthesis of the cDNAs, represented as dotted bars with their expected sizes in nucleotides on the left.

ing to the first residue of the conserved CUGA sequence of the (+)-strand hammerhead structure, *sub*-ASBVd(−) could have its 3′ end at nt 172, which corresponds to the first residue of the same conserved tetranucleotide in the (−)-strand hammerhead structure (Fig. 2*A*). If that were the case, *sub*-ASBVd(−) RNA would have a size of 148 nt, slightly larger than that of *sub*-ASBVd(+) (Fig. 1). To obtain evidence for this assumption, a mixture of these two RNAs was eluted from a preparative urea gel. Reverse transcription of this mixture using primer III gave the cDNA of 136 nt expected for *sub*-ASBVd(+) RNA (Fig. 2*A*), whereas that made with a 33-nt primer identical to ASBVd nt 172–204 gave a cDNA product with the size postulated for *sub*-ASBVd(−) RNA (data not shown). Moreover, the 5′ end of *sub*-ASBVd(−) was mapped at nt 69 from comparisons of the mobility of the latter cDNA with the ladders obtained by sequencing an ASBVd clone using the same primer and chain-terminating inhibitors (data not shown). These results support the notion that the *in vitro* self-cleavage reaction of ASBVd (−)-strand RNAs is also active *in vivo*.

Identification of *sup*-ASBVd(+) as a Fusion Product of Monomeric and Subgenomic (+)-Strand RNAs. The *sup*-ASBVd(+) RNA (Fig. 1*A*) was obtained from urea/0.25× TBE gels which allowed the isolation of this molecule free from other ASBVd RNAs. Since *sup*-ASBVd(+) RNA displayed a mobility consistent with that of a linear molecule having a length equal to the sum of monomeric and *sub*-ASBVd(+) RNAs (Fig. 1), primer extension analyses were performed to verify this hypothesis. Fig. 2*B* shows the major cDNAs expected to appear. The experimental results were consistent with this assumption (data not shown). Primer II generated a band of 249 nt, and primer III gave three bands with the mobilities expected for cDNAs of 136, 247, and 383 nt. Moreover, when the extensions with primer III were carried out in the presence of chain-terminating inhibitors, the sequence deduced for *sup*-ASBVd(+) was the same as that of *mc*-ASBVd(+).

Detection of *mc*-ASBVd(−) RNA Forming Part of Multistranded Complexes. Fig. 3 shows the ASBVd RNAs detected in Northern blots of nondenaturing gels hybridized with ASBVd probes. Three main bands were observed with the probe for ASBVd (+)-strand RNAs (Fig. 3*A*, lane 2), which should be produced predominantly by the dimeric (*d*), monomeric (*m*), and *sub*-ASBVd(+) RNAs, since those were the mobilities of the corresponding three RNAs purified from urea gels (circular and linear forms migrate as a single band in nondenaturing gels). Other prominent bands were detected in the upper part of the gel (*y*), and between the *m*- and *d*-ASBVd RNAs (*x*) (Fig. 3*A*, lane 2). The same *m*, *d*, *x*, and *y* bands were also seen in the Northern blot hybridized with the probe for ASBVd (−)-strand RNAs (Fig. 3*B*, lane 2). Interestingly, whereas bands corresponding to *d*, *m* and *sub*-ASBVd(+) RNAs displayed very attenuated intensities in the Northern blot for ASBVd (−)-strand RNAs, the decrease in the intensities of bands *x* and *y* was less pronounced, suggesting that they contained a significative proportion of ASBVd (−)-strand RNAs. Moreover, when the nucleic acids were denatured prior to gel application, bands *x* and *y* disappeared (Fig. 3, lanes 3), whereas bands *m* and *d* displayed increased intensities, particularly in the Northern blot hybridized with the probe for ASBVd (−)-strand RNAs (Fig. 3*B*, compare lanes 2 and 3), indicating that bands *x* and *y* were generated by multistranded complexes containing monomeric and dimeric ASBVd forms of both polarities.

Chromatography on cellulose in STE containing 25% ethanol provided additional evidence about the nature of bands *x* and *y*. Most of the RNAs giving rise to *sub*-ASBVd(+), *m* and *d* bands did not bind to cellulose, whereas the RNAs generating bands *x* and *y* bound preferentially to cellulose and were then released with STE (data not shown), indicating that these two latter bands contained multistranded complexes with extended double-stranded regions. To investigate these complexes in more detail, the nucleic acids present in bands *x* and *y*—as well as in bands *m* and *d*, taken as controls—were extracted and analyzed by urea/PAGE and Northern blot hybridization. Band *m* gave signals in the positions of *mc*- and *ml*-ASBVd RNAs, but those obtained with the probe for ASBVd (+)-strand RNAs were much more intense (Fig. 4, lanes 3), showing that band *m* was composed essentially of monomeric ASBVd(+) RNAs. Band *x* also gave two main signals in the positions of *mc*- and *ml*-ASBVd RNAs, but their intensities were similar with both probes (Fig. 4, lanes 4), indicating the existence of both (+) and (−) *mc*-ASBVd

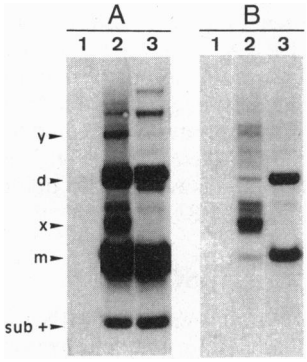

FIG. 3. Northern blot hybridizations of avocado nucleic acids after nondenaturing PAGE, using RNA probes for (+) (*A*) and (−) (*B*) ASBVd sequences. Lanes 1 and 2, nucleic acids from uninfected and ASBVd-infected avocado leaves, respectively; lanes 3, the same extracts as in lanes 2, but before loading the samples were boiled at 100°C for 1.5 min in 8 M urea and then quickly cooled on ice. Arrowheads indicate dimeric (*d*), monomeric (*m*) and subgenomic (*sub*+) ASBVd (+)-strand RNAs, as well as two other prominent ASBVd-specific bands (*x* and *y*).

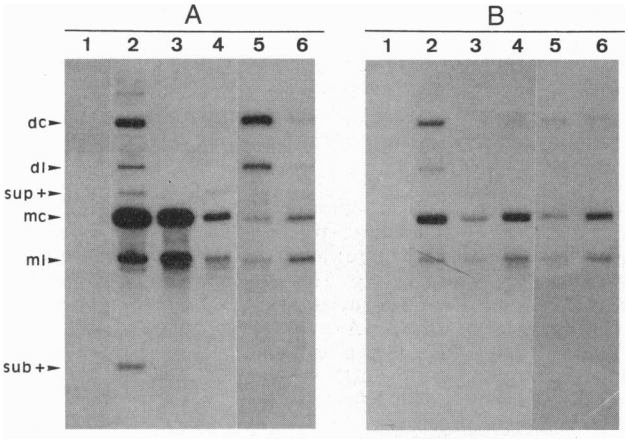

FIG. 4. Northern blot hybridizations of avocado nucleic acids extracted from several regions of a nondenaturing gel. After extraction the RNAs were denatured, separated in a gel with urea, and probed with radioactive RNAs for (+) (*A*) and (−) (*B*) ASBVd sequences. Lanes 1 and 2, nucleic acids from uninfected and ASBVd-infected avocado leaves, respectively; lanes 3–6, nucleic acids extracted from regions *m*, *x*, *d*, and *y*, respectively, of a nondenaturing gel. Other details are as in the legend to Fig. 1.

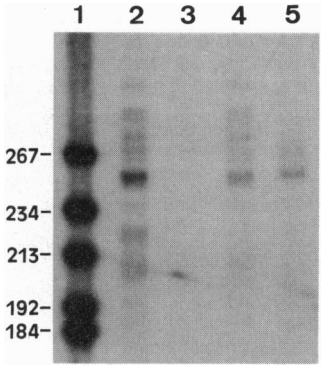

FIG. 5. Primer extension analyses of *mc*-ASBVd RNAs from bands *m* and *x* of a nondenaturing gel. After extraction the RNAs were denatured as in Fig. 1 and applied to a gel with urea. The *mc*-ASBVd RNAs were then eluted and reverse transcribed with primers III and IV of (−) and (+) polarities, respectively. Lane 1, radioactive DNA markers with their sizes in nucleotides on the left; lanes 2 and 3, cDNAs obtained with primers III and IV, respectively, and the *mc*-ASBVd RNA from band *m*; lanes 4 and 5, cDNAs obtained with primers III and IV, respectively, and the *mc*-ASBVd RNA from band *x*. In overexposed autoradiograms, cDNAs longer than unit length were also observed in lane 5.

RNAs in similar proportions. Band *d* gave main signals in the positions of *dc*- and *dl*-ASBVd RNAs, but those obtained with the probe for ASBVd (+)-strand RNAs were more intense (Fig. 4, lanes 5), showing that the major components of band *d* were the (+)-strand dimeric ASBVd RNAs. Band *y* generated main signals in the positions of *mc*- and *ml*-ASBVd RNAs (Fig. 4, lanes 6) whose intensities were similar with both probes, indicating that band *y*, like band *x*, must contain both (+) and (−) *mc*-ASBVd RNAs in approximately the same proportions. Also worthy of note are the minor components of bands *d* and *y*, which include (−) and (+) *mc*- and *ml*-ASBVd RNAs in similar ratios in band *d* (Fig. 4, lanes 5) and (+) and (−) *dc*-ASBVd RNAs also in similar ratios in band *y* (lanes 6).

To obtain definitive evidence on the existence of *mc*-ASBVd(−) RNA, the RNAs of bands *m* and *x* from nondenaturing gels were eluted, denatured, and separated in urea gels. From these gels, the *mc*-ASBVd RNA band again was eluted and the nature of the RNA was established by primer extension analyses using primers III and IV of (−) and (+) polarities, respectively. With *mc*-ASBVd RNA from band *m*, a prominent unit-length cDNA was obtained with primer III but not with primer IV (Fig. 5, lanes 2 and 3). Conversely, with *mc*-ASBVd RNA from band *x*, unit-length cDNAs of similar intensities were observed with both primers (Fig. 5, lanes 4 and 5). The unit-length cDNAs were accompanied by other longer-than-unit products expected to appear from the transcription of circular templates (Fig. 5, lanes 2, 4, and 5). These results showed unambiguously that band *x* contained (+) and (−) *mc*-ASBVd RNAs.

DISCUSSION

The identification of *mc*-ASBVd(−) RNA is direct evidence that ASBVd, unlike potato spindle tuber viroid and probably the rest of the known viroids with the exception of peach latent mosaic viroid, replicates through a symmetric pathway. Other small infectious RNAs following this strategy are the satellite RNA of tobacco ringspot virus (14) and probably some other satellite RNAs from nepo-, sobemo-, and luteoviruses (13, 14), as well as a small circular RNA from carnation (30), all of which display autolytic processing in their RNAs of both polarities. Moreover, the detection of *mc*-ASBVd(−) RNA in multistranded complexes also favors a template role for it. Bands *x* and *y* also contain *mc*-ASBVd(+) RNA, an observation also consistent with a symmetric pathway.

The characterization of the 5′ termini of *sub*-ASBVd(+), *sub*-ASBVd(−), and *sup*-ASBVd(+) RNAs has revealed that they are identical to the 5′ termini generated in the *in vitro* self-cleavage reactions of the respective (+) and (−) dimeric ASBVd RNAs (11). These results support the involvement of the *in vitro* reactions in the *in vivo* replication of ASBVd and confirm previous observations on the 5′ end of a fraction of the linear monomeric plus RNA of ASBVd isolated from infected tissue (28).

An extra cytidylate was found at the 3′ end of *sub*-ASBVd(+) RNA, and presumably a similar terminus is present on *sup*-ASBVd(+). The cytidylate could result from the incorporation of a nontemplate-encoded 3′ residue. Unpaired extra guanidylates at the 3′ ends of the (−)-strands of the double-stranded RNAs of two satellite RNAs from cucumoviruses have been detected (31). It is also possible that prior to the addition of the extra cytidylate, the 3′ termini of *sub*-ASBVd(+) and *sup*-ASBVd(+) may have suffered some processing. It is difficult to define the termination point of an RNA synthesized *in vivo*, since it is possible that the 3′ end of the molecule has been generated by cleavage of the transcript and, therefore, does not represent the actual site at which RNA polymerase terminated (32). If such processing has not occurred, several alternatives could explain why RNA synthesis terminates around nt 191 in *sub*-ASBVd(+) and *sup*-ASBVd(+). First, during elongation of the (+)-strand, the (−) template could adopt a local folding causing the polymerase to dissociate in some of the replicative rounds. One such folding, a hairpin whose stem is formed by part of the conserved sequences of both hammerhead structures (see Fig. 2*A*), is presented (Fig. 6 *Left Inset*).

Second, some eukaryotic terminators result from the formation of a hairpin in the RNA being transcribed, indicating that termination depends on the RNA product (32). An element of secondary structure can be formed by the nascent (+)-strand just preceding nt 191 (Fig. 6 *Right Inset*). Third, termination may also be due to RNA tertiary structures or obstruction by proteins. In these first three alternatives, a unique 3′ end would result from premature termination of (+)-strands.

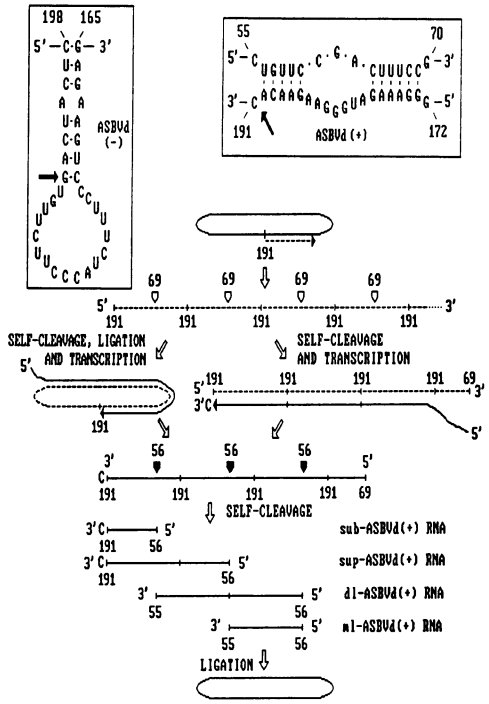

FIG. 6. Models for the generation of *sub*-ASBVd(+) and *sup*-ASBVd(+) RNAs. The same numbers are used in the (−) polarity as in the (+) polarity. The steps leading to *sub*-ASBVd(+), *sup*-ASBVd(+) and other ASBVd(+) RNAs are shown. The C on the left of *sub*-ASBVd(+), *sup*-ASBVd(+) and the (+) oligomeric transcript RNAs refers to the extra cytidylate of *sub*-ASBVd(+) RNA, possibly also present in the other species as well. Continuous and broken lines indicate (+) and (−) polarities, respectively, and solid and open arrowheads indicate self-cleavage sites of ASBVd (+)- and (−)-strands, respectively. (*Left Inset*) Hairpin which can be adopted by the ASBVd (−) template. The stem of this hairpin is formed by sequences conserved in the (+) and (−) hammerhead structures. (*Right Inset*) Element of secondary structure which can be formed by the nascent (+)-strand. Arrows in *Insets* indicate the position where synthesis of ASBVd (+)-strand RNA terminates.

A fourth alternative is to consider the specific initiation of (−)-strands around nt 191 of the (+)-strand circular template (Fig. 6). Self-cleavage of the (−) oligomers at two adjacent sites would generate unit-length RNA, and self-cleavage at only one site would produce a series of RNAs having common 3′ termini. These termini could be recognized by a polymerase catalyzing the synthesis of (+) oligomeric RNAs which, after processing by one of the two self-cleavage sites proximal to their 3′ termini, would generate *sub*-ASBVd(+) and *sup*-ASBVd(+) RNAs; monomeric and dimeric (+)-strand RNAs would result from autolytic processing by other alternative sites, producing after ligation the major terminated products. However, in this alternative a significant fraction of the templates for synthesis of (+)-strands should be the (−) oligomers and not the (−) monomeric circular RNA detected forming complexes with the (+)-strands.

The high self-complementarity of *sub*-ASBVd(+), *sub*-ASBVd(−), and *sup*-ASBVd(+) RNAs probably contributes to protecting them from degradation by cellular RNases. We now interpret our failure in sequencing *sub*-ASBVd(+) RNA with base-specific RNases (data not shown) as a consequence of its compact folding. On the other hand, *sup*-ASBVd(+) is not expected to self-cleave, since it cannot form the double-hammerhead structure proposed to mediate the self-cleavage of ASBVd (+)-strand RNAs (33). The extension of the approach used here for ASBVd to other small infectious RNAs from plants should help to better understand their replication. There are indications that the situation found in ASBVd may not be unique, since RNAs with a size not a multiple of the unit length have been also detected in tissues infected by some circular satellite RNAs with self-cleavage domains (5).

We thank Dr. R. H. Symons for the initial ASBVd clone, Dr. J. M. Farré for avocado material, and V. Moncholí and M. Climent for technical assistance. We thank Dr. H. L. Sänger for suggestions and D. Wilkes and H. S. Padgett for revision of the English text. This work has been partially supported by Grants PB87-0346 and PB92-0038 from the Dirección General de Investigación Científica y Técnica of Spain and by predoctoral fellowships from the Ministerio de Educación y Ciencia (to J.A.D. and J.F.M.) and from the Generalitat Valenciana (to C.H.).

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