Oligomers of avocado sunblotch viroid are found in infected avocado leaves

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1. INTRODUCTION

The most abundant form of avocado sunblotch viroid (ASBV) in extracts of infected leaves of avocado (*Persea americana*) is a single-stranded covalent RNA circle [1] of 247 residues [2]. Highly purified preparations of the circular viroid infect avocado and induce the sunblotch disease [3]. ASBV has a limited sequence homology with 3 other viroids which have extensive sequence homology with each other [2,4]; potato spindle tuber viroid (PSTV), chrysanthemum stunt viroid (CSV) and citrus exocortis viroid (CEV). The level of ASBV in nucleic acid extracts from leaves of infected avocado leaves from different trees varied over a 10 000-fold range [5], while there was a 1000-fold variation in the concentration of ASBV in extracts prepared from 6 branches of the same tree [6].

We report here that extracts of leaf tissue from strongly infected avocado seedlings contain an oligomeric series of RNAs which are integral multiples of the unit length ASBV and are of the same (+) polarity. Complementary (−) RNAs are also present but in much lower concentrations.

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2. MATERIALS AND METHODS

Leaves of avocado seedlings infected with the SB-1 strain of ASBV [1,2] were kindly supplied from glasshouse stocks by D. McE. Alexander (CSIRO Division of Horticultural Research, Merbein VIC). Circular and linear forms of ASBV were purified as in [1,5,7]. Partially purified nucleic acid extracts of avocado leaves, to be used for analysis by gel electrophoresis, were prepared as in [5,6] with the modification that, after the initial homogenization, addition of solid NaCl and stirring at room temperature for 30 min, the extract was then centrifuged without prior cooling on ice for 30 min. Nucleic acid samples were incubated in 1 M glyoxal, 10 mM sodium phosphate (pH 6.5) at 50°C for 1.0 h [8] and then electrophoresed on 1.9% agarose slab gels (15 × 14 × 0.3 cm) in 10 mM sodium phosphate (pH 6.5) at 30 mA. After the xylene cyanol FF marker dye had migrated 5.0 cm, nucleic acids were transferred by blotting to cellulose nitrate sheets which were baked at 80°C in vacuo for 3–4 h prior to hybridisation (see below) [9].

ASBV was initially cloned into the plasmid vector pBR322 and then into the single-stranded vector M13mp93. Thus, double-stranded cDNA was prepared from purified, linear ASBV after removal of any 3′-terminal phosphate, essentially as described in [10] for the viroid-like RNA of solanum nodiflorum mottle virus. Double-stranded cDNA termini were made blunt by treatment with S_1 nuclease followed by incubation with the 4 dNTPs

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and Klenow fragment of *E. coli* DNA polymerase I [11]. The vector pBR322 was cut with *Sal*I, its termini filled with the 4 dNTPs using Klenow fragment of DNA polymerase, treated with calf intestinal alkaline phosphatase and recovered by ethanol precipitation after phenol–chloroform deproteinization [11]. The vector and double-stranded cDNA were ligated with T_4 DNA ligase and used for transformation of *Escherichia coli*. Colonies which were Amp$^+$ Tet$^-$ were screened by colony hybridization [12] using a c[^{32}P]DNA probe to ASBV [1,5]. Cloned inserts were removed from purified plasmids by digestion with *Taq*I, their termini filled, ligated to *Eco*RI linkers [13] and inserted into the *Eco*RI site of the bacteriophage vector M13mp93 [Messing, personal communication]. Phage stocks were raised from single white plaques, the DNA isolated and the cloned inserts sequenced by the Sanger dideoxynucleotide chain termination technique [14,15] using the specific M13 primer, $TC_3AGTCACGACGT$ (New England Biolabs, Waltham MA).

Clones containing either a + or − insert corresponding to residues 81–223 of ASBV [2] were used for the preparation of single-strand c[^{32}P]-DNA probes for the detection of + and − RNA sequences, respectively. Thus, the specific M13 15-mer primer was hybridized to each clone and extended using the Klenow fragment of DNA polymerase I in the presence of d[α-^{32}P]CTP (spec. act. 1000 Ci/mmol, [16]) and the other 3 unlabelled dNTPs. After a chase of 0.1 mM dCTP, the product was cut at the single *Hin*dIII site, denatured by heating at 100°C for 3 min in 35% (v/v) formamide and the fragments fractionated by electrophoresis on a thin 5% polyacrylamide gel in 90 mM Tris–borate (pH 8.3), 2 mM EDTA, 7 M urea [17]. After brief autoradiography, the single-strand c[^{32}P]DNA band with R_f of 0.8 relative to the xylene cyanol FF marker dye was cut out, eluted from the gel slice overnight at 37°C [18] and concentrated by ethanol precipitation in the presence of carrier tRNA. The + and − probes were dissolved in 10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, 0.1% SDS, 5 mM 2-mercaptoethanol, and used for hybridization to RNA sequences transferred to cellulose nitrate sheets from agarose gels [9]. Prehybridization at 37°C and hybridization with the [^{32}P]DNA probes at 55°C were done essentially as in [9] but the cellulose nitrate strips, after hybridization, were washed 4 times at room temperature with 2 × SSC, 0.1% SDS, and then twice for 30 min at 55°C with 0.1 × SSC, 0.1% SDS, prior to autoradiography.

3. RESULTS

3.1. *Circular dimers of ASBV can be isolated from infected avocado leaves*

The fractionation of partially purified nucleic acid extracts of avocado leaves infected with ASBV by electrophoresis under native conditions on a 5% polyacrylamide slab gel gave 2 stained nucleic acid bands (fig.1A), not seen when extracts from healthy leaves were electrophoresed under the same conditions [1]. The faster moving of these 2 bands was present in much greater amounts and was the unit length ASBV (extensively characterized in [1,2]). The slower moving band ran in the approximate position expected for a dimer of ASBV. Both bands were electroeluted [19] and electrophoresed on a 5% polyacrylamide slab gel under denaturing conditions (7 M urea). As observed under these conditions in [1,5,7], ASBV was separated into a major band of circular ASBV and a faster moving minor band of linear ASBV (track 2, fig.1B). Band X was not identified. The putative dimer ASBV showed similar properties (track 1, fig.1B): the slower moving major band was taken as the circular form; the faster band as the linear dimer.

The size of the putative ASBV dimer was determined by electrophoresis of glyoxalated RNA in the presence of glyoxalated M_r-markers on a 1.9% agarose gel in 10 mM sodium phosphate buffer (pH 6.5) (fig.2); under these conditions, linear ASBV migrated slightly slower than circular ASBV (see below). The markers used were purified coconut cadang cadang viroids (CCCV), CCCV RNA 1 fast (isolate Baao 54, 246 residues) and CCCV RNA 2 fast (492 residues) [20], and single-strand restriction enzyme *Hpa*II fragments of the replicative form of bacteriophage M13mp7 [21], labelled by filling its termini with the Klenow fragment of DNA polymerase I in the presence of d[α-^{32}P]CTP and d[α-^{32}P]GTP [11]. The putative dimer ASBV migrated in the position expected for a dimer of 494 residues (fig.2).

Final evidence that the putative dimer of ASBV was a true sequence dimer was shown by two-

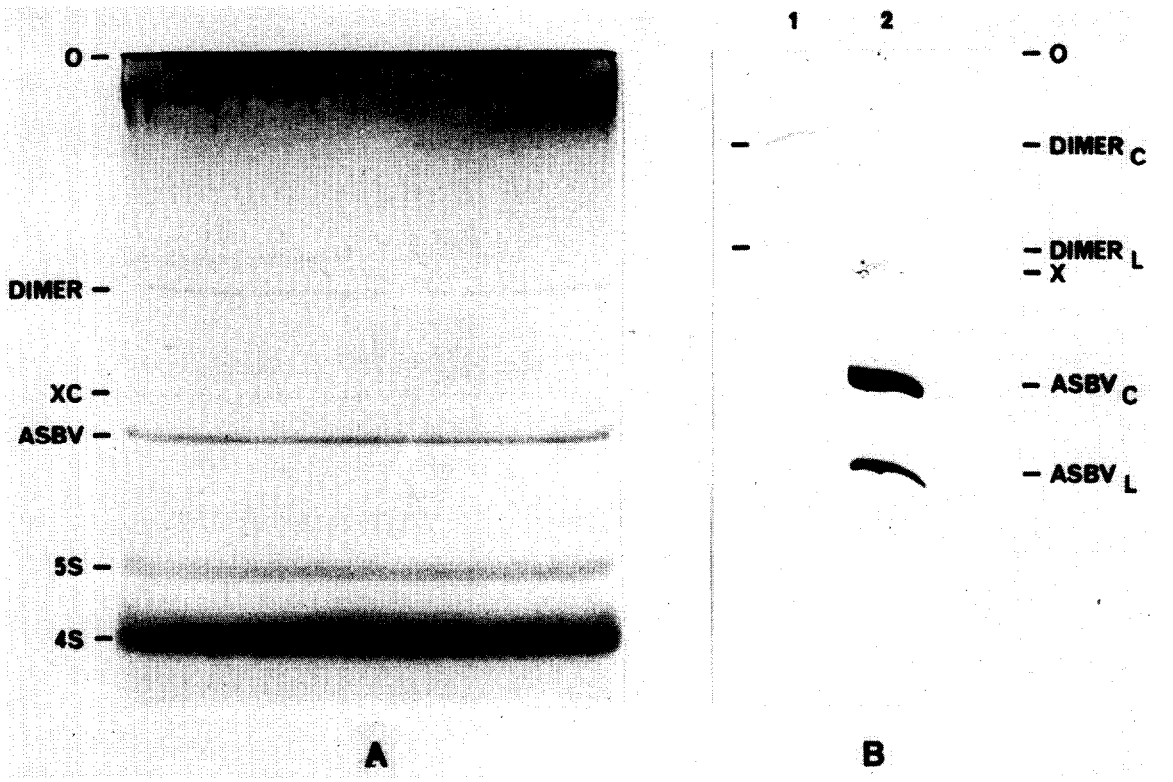

Fig.1. Analysis of extracts of ASBV-infected avocado leaves by polyacrylamide gel electrophoresis. (A) Partially purified nucleic acid extract from 35 g leaves was electrophoresed on a 16×16×0.3 cm 5% polyacrylamide gel in Tris–sodium acetate–EDTA buffer [7,19] at 25 mA for 16 h. The gel was stained for 10 min in 0.02% toluidine blue and destained in water. Positions of the dimer ASBV, xylene cyanol FF marker dye, ASBV and 5 S and 4 S RNA are given. (B) Dimer and ASBV bands from A were electroeluted [19] and run on a 90 mM Tris–borate, 2 mM EDTA, 7 M urea, 5% polyacrylamide gel (20×40×0.05 cm) at 20 mA for 1.8 h. The gel was stained with 10 μg ethidium bromide/ml for 30 min, destained in water and photographed under ultraviolet light. The positions of the circular and linear forms of the dimer ASBV, ASBV and an unidentified band X are given. The mobility of circular ASBV was 0.4 relative to the xylene cyanol FF marker dye.

dimensional fingerprinting of 5′-^{32}P-labelled fragments in complete ribonuclease A digests [22] of both ASBV and the putative circular dimer; identical patterns were obtained (not shown).

3.2. *Preparation of [^{32}P]DNA probes for the analysis of ASBV sequences in extracts of avocado leaves*

The most difficult aspect of this work was the preparation of [^{32}P]DNA probes which would each give an unequivocal demonstration of the presence of either ASBV (+) sequences or complementary (−) sequences. This required that, on hybridisation, [^{32}P]DNA probes for + sequences did not hybridize at all with − RNA sequences and vice versa. This problem was accentuated by the observation that − sequences were present at much lower concentrations in leaf extracts than + sequences (see below). Probes prepared from purified ASBV by 5′-^{32}P-labelling of partial ribonuclease digests [2] or by reverse transcription of linear, polyadenylated fragments of ASBV [1,5] cannot be used because of the possible presence of contaminating host RNA fragments and because purified ASBV may contain trace amounts of − sequences of the same size. Hence, [^{32}P]DNA probes were derived from cloned fragments of ASBV in the single-strand phage M13mp93 vector; these clones contained a 143 residue sequence of ASBV (residues 81–223; [2]) as either the + or −

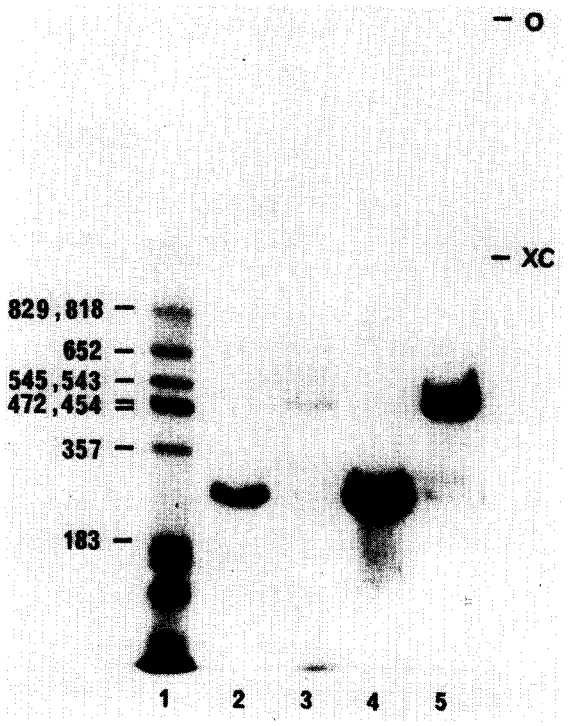

Fig.2. M_r-value estimation of ASBV dimer. Glyoxalated RNA and DNA samples were electrophoresed on a 1.9% agarose gel (14×14×0.3 cm) in 10 mM sodium phosphate (pH 6.5) at 30 mA until the xylene cyanol FF marker migrated 5.0 cm. The nucleic acids were transferred to cellulose nitrate and hybridized [9] with a mixture of [^{32}P]DNA probes to ASBV and coconut cadang cadang viroids [20] (supplied by J. Haseloff): (1) *Hpa*II digest of a replicative form of phage M13mp7, its termini filled with d[α-^{32}P]CTP (supplied by J. Haseloff); lengths of the single-stranded fragments are given; (2) circular ASBV; (3) circular dimer ASBV; (4) CCCV RNA 1 fast, 246 residues; (5) CCCV RNA 2 fast, 492 residues.

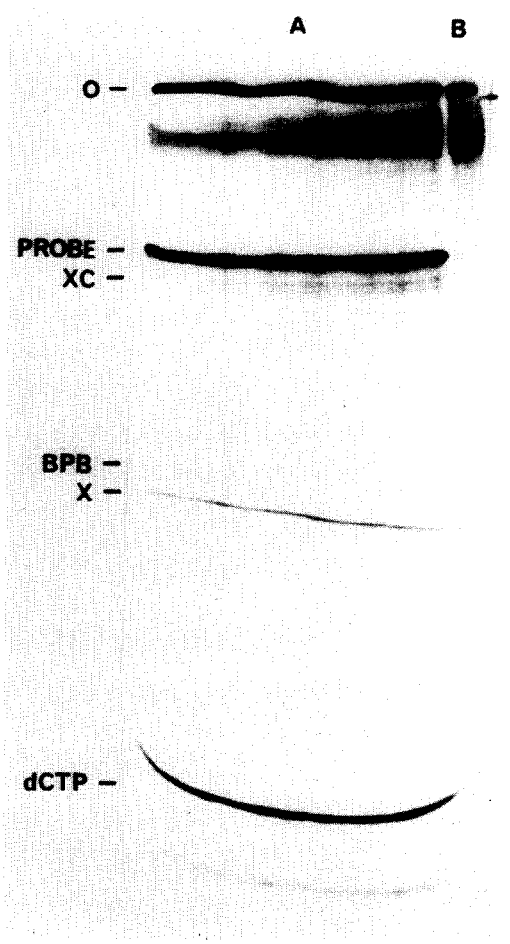

Fig.3. Purification of single-stranded [^{32}P]DNA probe for + sequences of ASBV. Reaction mixture, prepared as in section 2, was electrophoresed on a 5% polyacrylamide gel (20×40×0.05 cm) at 30 mA 1.2 h in 90 mM Tris–borate (pH 8.3), 2 mM EDTA, 7 M urea, then autoradiographed for 2 min: (A) reaction mixture in 35% formamide denatured by heating at 100°C for 3 min prior to loading on gel; (B) sample of reaction mixture not heated prior to loading. *Abbreviations*: O, origin of gel; probe, position of the single-strand DNA probe; XC and BPB, position of the marker dyes, xylene cyanol FF and bromophenol blue; X, unidentified material; dCTP, unincorporated d[α^{32}P]CTP.

sequence in the *Eco*RI site of M13mp93.

Suitable single-strand [^{32}P]DNA probes were prepared by transcription across the cloned insert using a synthetic 15-mer DNA fragment: as primer in the presence of d[α-^{32}P]CTP and the Klenow fragment of DNA polymerase I; cutting the double-stranded product with *Hin*dIII 30 residues downstream from the terminus of the cloned insert; and purification, after denaturation of the double-stranded DNA from the single-stranded [^{32}P]DNA probes, by polyacrylamide gel electrophoresis in the presence of 7 M urea. An autoradiogram of a gel used for the preparation of the + [^{32}P]DNA probe (detects + ASBV sequences) is given in fig.3A; an identical pattern was obtained for the – probe. Because M13mp93 only has one *Hin*dIII site, the products of *Hin*dIII digestion are the single-stranded [^{32}P]DNA probe

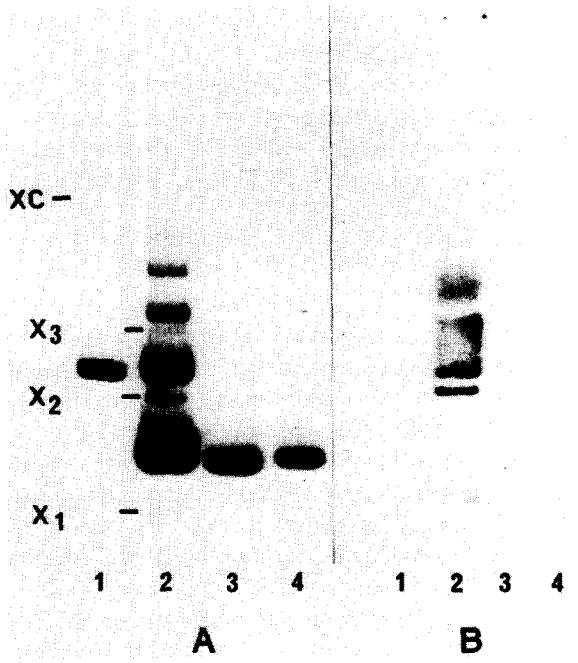

Fig.4. Detection of ASBV + and – sequences in nucleic acid extracts of infected avocado leaves after electrophoresis in a 1.9% agarose gel (14×14×0.3 cm) in 10 mM sodium phosphate (pH 6.5) at 30 mA for 3.0 h. Nucleic acid samples were glyoxalated before electrophoresis. After transfer to cellulose nitrate sheet, nucleic acids were probed with single-stranded [^{32}P]DNA probes for + ASBV sequences (A) or for – ASBV sequences (B). Autoradiography was for 6 h at room temperature in (A) and for 4 days at –70°C in presence of an intensifying screen in (B): (1) marker circular dimer ASBV; (2) nucleic acid extract; (3) marker circular ASBV; (4) marker linear ASBV; bands X_1–X_3, see text; XC, xylene cyanol FF marker dye, 5.0 cm from origin.

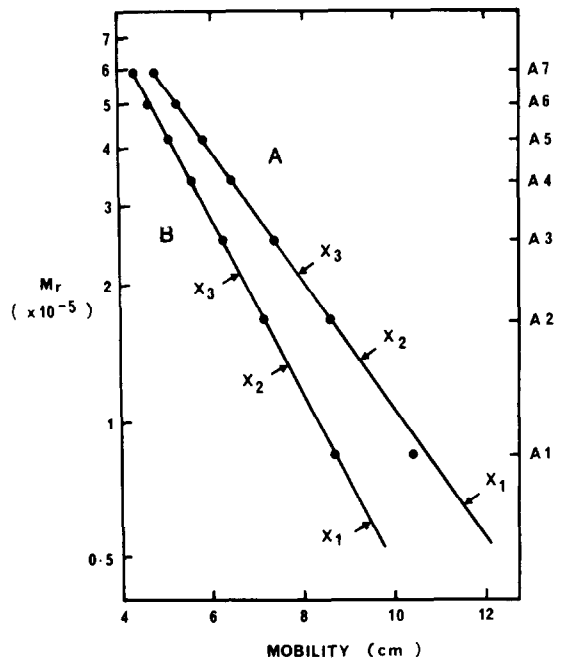

Fig.5. Plot of the logarithm of the presumed M_r-values of the oligomeric series of + ASBV bands against mobility. Oligomeric ASBV series, A_1–A_7, based on M_r 84 000 for ASBV [2]. (A) Data from fig.4A; (B) data from another experiment, for fig.4A but RNA samples were pretreated with formaldehyde and electrophoresed on a 1.9% agarose gel in 2 M formaldehyde [28,29]. Positions of bands X_1–X_3 on each gel are indicated.

(fig.3A), long [^{32}P]DNA fragments complementary to M13mp93 downstream from the cloned insert and linear, unlabelled M13mp93; the latter 2 run at the top of the gel in fig.3A because of their size. The minor band X has not been identified while the lower band in fig.3A is the unincorporated d[α-^{32}P]CTP. Fig.4B is a sample of the reaction mixture which was not denatured at 100°C prior to electrophoresis; essentially all the labelled DNA is present as a high-M_r, double-stranded complex.

3.3. *Analysis of + and – sequences of ASBV in extracts of infected leaves*

Partially purified nucleic acid extracts of ASBV-infected avocado leaves were reacted with glyoxal to ensure denaturation of all component nucleic acids. After fractionation by electrophoresis on 1.9% agarose gels in 10 mM sodium phosphate (pH 6.5), nucleic acids were transferred by blotting to nitrocellulose sheets and immobilized by baking at 80°C in vacuo for 3–4 h [9]; baking for longer than the usual 2 h was done to ensure breakdown of the glyoxal–nucleic acid complexes. Duplicate sheets were then hybridized with single-stranded [^{32}P]DNA probes prepared against M13mp93 clones containing either + or – ASBV sequences.

The results of one experiment are given in fig.4. The [^{32}P]DNA probe for + RNA sequences showed a series of bands of increasing M_r-value with the two fastest migrating bands corresponding to the monomer and dimer circular ASBV markers (fig.4A). That the probe hybridized to RNA was shown by the sensitivity of the complex to pan-

creatic RNase (not shown). The series of RNA bands which decreased in intensity with size are taken as oligomers of the monomer ASBV (M_r 84 000, [2]) since a plot of the mobility of each band against the logarithm of its putative M_r-value was linear (fig.5, line A). Although only oligomers of up to 5-times the unit length can be seen in fig.4A, bands equivalent to 8-times the unit length are visible on the original autoradiogram. This oligomeric pattern of ASBV sequences was also seen when nucleic extracts of infected leaves were analysed by electrophoresis on agarose gels in the presence of 2 M formaldehyde [28,29]. A plot of the log M_r of the 7 oligomers *vs* mobility is given for one experiment in fig.5, line B.

A minor series of 3 bands (X_1, X_2 and X_3 in fig.4A) were always seen in nucleic acid extracts of infected leaves. On the basis of the plots of fig.5, estimated M_r-values were 65 000, 135 000 and 210 000. However, the nature of these ASBV + RNAs is not known.

As isolated, ASBV monomers and dimers existed mostly in the circular form (fig.1B and other data) but the relative distribution of circular and linear forms in the higher oligomers is not known. Careful analysis of a number of autoradiograms similar to fig.4A has indicated the presence of 2 closely migrating bands in these higher oligomers; it is feasible that these 2 bands represent the circular and linear forms of each oligomer since there was a small difference in the mobilities of linear and circular ASBV, with circular ASBV migrating faster than the linear form (fig.4A).

In contrast to the results with the [^{32}P]DNA probe for + ASBV sequences, the probe for – sequences showed the presence of low levels of sequences complementary to ASBV (fig.4B). The main – species were 2 closely migrating bands with mobilities similar to that of the ASBV dimer (fig.4A). In addition, there were 2 minor bands with mobilities slightly less than the ASBV monomer and a blur of high-M_r material (fig.4B). Most importantly, there was no hybridization of the – probe to the markers of linear, circular and dimer ASBV (fig.4B) which showed that the stringent washing conditions used for the cellulose nitrate strips (0.1 × SSC, 0.1% SDS, at 55°C for 1.0 h) were sufficient to eliminate any hybridization of DNA probes to RNA sequences of the same polarity. The total radioactivity hybridized using the + and – probes was determined by counting the cellulose nitrate strips of track 2 in fig.4A,B; track 2 in fig.4B contained 0.5% of the cpm in track 2 in fig.4A, and this gives an approximate estimate of the relative amounts of – and + ASBV sequences, respectively, since the 2 probes had the same specific radioactivity.

4. DISCUSSION

This is the first report of an oligomeric series of + viroid sequences in extracts of viroid-infected tissue. Greater than unit length RNAs complementary to PSTV [23–25] and to CEV [26] have been detected by their hybridization to viroid RNA or to specifically labelled cloned DNA. Apparently, sequences complementary to all of the sequences of PSTV are represented in the – RNA from PSTV-infected tissues [27]. As shown here, – sequences are mostly of dimer length or smaller with a small amount of higher-M_r material (fig.4B); these – sequences were ~0.5% of the total + sequences. In [25], no multimeric PSTV molecules were detected in infected tomato tissue. However, we have repeatedly observed the oligomeric pattern of + sequences of fig.4A during the past year using a range of [^{32}P]DNA probes prepared from phage M13 clones of ASBV and from purified ASBV [1,5] as well as using different sources of infected avocado leaves to the one used here (not shown).

Although evidence that the oligomers of ASBV observed here are intermediates in replication (rather than end products) is lacking, it is attractive to consider that they originated because of a 'rolling circle' mechanism of ASBV replication. Thus, invading monomeric ASBV could be converted by host enzymes to a – circular molecule which then acts as the template for the rolling circle synthesis of a continuous + ASBV sequence which is processed by specific ribonucleases to give full-length linear monomeric ASBV. Ligation then produces the predominant monomer circular ASBV. The oligomeric series of ASBV could arise if the processing did not keep pace with the synthesis of long linear ASBV and the dimers, trimers, etc. were not subject to further processing once removed from the site of replication. This simple model does not account for the minor oligomeric series of + molecules (X_1, X_2 and X_3) seen in

fig.4A nor for the, as yet uncharacterized, – ASBV structures.

Multimeric, linear, single-stranded RNAs of + polarity have been found for the encapsidated satellite RNA of tobacco ringspot virus (TRSV) [29]. Further, when double-stranded RNA isolated from TRSV-infected tissue was denatured, an oligomeric series of both + and – sequences of satellite RNA was found and a rolling circle model of transcription suggested for the origin of these RNAs [29].

It is important to stress that cloned single-stranded DNA probes are essential for the unequivocal demonstration of + and – RNA sequences in this type of work. The above method has provided [^{32}P]DNA probes of the required specificity and of high specific activity ($5-10\times10^8$ cpm/μg). Cloned, double-stranded DNA probes in which one of the two strands is labelled cannot be used. For example, we originally prepared labelled DNA probes by transcription across the cloned ASBV insert in the *Acc*I site of the vector M13mp7 [21] using the synthetic 15-mer M13 primer. The double-stranded product was cut with *Eco*RI, which cleaves on either side of the cloned insert, and the double-stranded fragment purified by gel electrophoresis. + and – labelled DNA probes were prepared using clones containing + and – ASBV sequences, respectively. However, when the – probe was used for hybridization (fig.4B) strong hybridization was obtained to produce a pattern identical to that found with the + probe (fig.4A). It appears that the hybridization of the – probe to + sequences was due to network formation of the two strands of the DNA probe in which the unlabelled – DNA strand hybridized in part to available sequences of ASBV bound to cellulose nitrate, the labelled + DNA strand then hybridized in part to the hybridized unlabelled – strand and so on to give a spurious result.

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