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2′ Phosphomonoester, 3′-5′ phosphodiester bond at a unique site in a circular viral RNA

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***Solanum nodiflorum* mottle virus (SNMV) RNA2 is a single-stranded, covalently closed circular molecule. RNase T2 or nuclease P1 digests of this RNA contain a minor nucleotide of unusual chromatographic and electrophoretic mobility. This nucleotide is resistant to further digestion by T2 or P1 ribonucleases, or by alkali, but is sensitive to venom phosphodiesterase digestion. Alkaline phosphatase digestion yields a product which is RNase T2 and P1 sensitive. The products of these various digests show that the minor nucleotide is a ribonuclease-resistant dinucleotide carrying a 2′ phosphomonoester group with the core structure $C^{2'}_{3'}p_{5'}A$. This dinucleotide is found in a unique RNase T1 product of SNMV RNA2, thus establishing a unique location in the sequence for the 2′ phosphomonoester group at residue 49. Identical results have been obtained with a second related virus. The phosphomonoester group probably results from the RNA ligation event by which the molecules were circularised.**

Key words: 2′ phosphomonoester/RNA ligase/RNA splicing/viroids/virusoids

Introduction

Solanum nodiflorum mottle virus (SNMV) and velvet tobacco mottle virus (VTMoV) are closely related small icosahedral plant viruses which each encapsidate a single-stranded covalently closed circular RNA (RNA2, of 378 or 366 bases, respectively) in addition to a linear genomic RNA (RNA1, of ~4.5 kb) (Randles *et al.*, 1981; Gould and Hatta, 1981; Haseloff and Symons, 1982). These small circular viral RNAs have attracted attention because they resemble viroids in certain features of their structure (circular topology and lack of conserved open reading frames) and mechanism of replication (appearance of concatameric forms of viral RNA in infected tissues) (Chu *et al.*, 1983; Symons *et al.*, 1984). They differ from viroids in being encapsidated and in their inability to infect plants without the large RNA (Gould *et al.*, 1981; Jones and Mayo, 1983). Viroid RNAs are infectious on their own as unencapsidated nucleic acid (Diener, 1971). Because of their resemblances to viroids, the name 'virusoids' has been suggested for encapsidated circular viral RNAs such as SNMV RNA2 and VTMoV RNA2. In relation to RNA1 they fulfil most if not all the criteria of satellite RNAs (for reviews, see Murant and Mayo, 1982; Francki, 1985).

It has been proposed that the concatameric forms of viroid RNA appearing in the course of replication are intermediates in a rolling circle mechanism of RNA synthesis (Branch *et al.*, 1981; Bruening *et al.*, 1982; Owens and Diener, 1982; Ishikawa *et al.*, 1984). Analogous models based on similar data have been proposed for virusoid RNA replication (Chu *et al.*, 1983; Symons *et al.*, 1984). There are some differences in detail among the various models, but they all require a final ligation step to circularise the progeny RNA. This seems likely regardless of the other details of the replication process since all known RNAs are initially synthesised as linear molecules.

RNA ligation reactions that have been studied in other contexts are known to proceed by a number of different mechanisms. In eukaryotes, all the RNA ligation steps that have been described occur in cellular RNA splicing pathways. Four such pathways have been worked out in some detail, each involving a distinct class of intron and variations in the reaction mechanism [nuclear tRNA splicing in yeast or plants, and in vertebrates, and rRNA splicing in *Tetrahymena* (see review by Cech, 1983), mammalian mRNA splicing (see Padgett *et al.*, 1984; Ruskin *et al.*, 1984)]. In addition, a class of intron structurally related to the *Tetrahymena* nuclear rRNA intron (Michel and Dujon, 1983; Waring *et al.*, 1983; Cech *et al.*, 1983) is widely dispersed in organelles, where two more classes of intron, whose mechanisms of excision have not yet been reported in detail, have also been defined on the basis of structural homologies [Class II introns (Michel and Dujon, 1983) and chloroplast mRNA introns (Koller *et al.*, 1984)]. Yet another kind of RNA ligation with some similarities to yeast and plant tRNA ligation (Uhlenbeck, 1983) is performed by a phage T4-encoded enzyme, which has been used to circularise linear virusoid RNA *in vitro* (Chu *et al.*, 1983). Since RNA ligation can proceed by many different mechanisms, it is clearly important to determine the mechanism which is used for virusoid RNA circularisation *in vivo*, both as a step towards understanding the overall mechanism of virusoid replication, and to clarify the resemblances which may exist between viroid and virusoid maturation and RNA splicing.

Here we show that ligation of virusoid RNA *in vivo* probably occurs by a mechanism known to be used by enzymes found in plants and fungi whose normal role is thought to be in nuclear tRNA splicing (Peebles *et al.*, 1979; Greer *et al.*, 1983; Gegenheimer *et al.*, 1983; Tyc *et al.*, 1983), but which (in the plant case) have been shown to be capable of circularising linear viroid RNA *in vitro* (Branch *et al.*, 1982; Kikuchi *et al.*, 1982). The characteristic signature of their reaction mechanism is the displacement of a 2′,3′ cyclic phosphate group at the 3′ end of an RNA by a 5′ phosphate group, leaving a 2′ phosphomonoester group beside the newly formed phosphodiester bond (Konarska *et al.*, 1981). Because of this 2′ phosphomonoester group the adjacent phosphodiester bond is resistant to alkali and most ribonucleases, and so the dinucleotide it bridges can be isolated by digesting the ligated RNA to end products. We have found such a dinucleotide in virusoid RNA, and mapped its position in the SNMV RNA2 molecule.

Results

Virusoid RNAs replicate to much higher titres than viroids in infected leaf tissue. Moreover, both SNMV and VTMoV will replicate in protoplasts from *Nicotiana clevelandii*, a systemic

host, as we have noted (Kiberstis and Zimmern, 1984). We have begun to study the replication of virusoid RNAs in this system, among the advantages of which is the feasibility of direct analysis of *in vivo* ^{32}P-labelled RNA. A ribonuclease T1 fingerprint of [^{32}P]SNMV RNA2 is shown in Figure 1a.

An unusual nucleotide in virusoid RNA

The position of digestion products on fingerprints is uniquely determined by their base composition. The smaller products of digestion form a regular pattern from which their base composition can be determined by inspection (Barrell, 1972). Inspection of fingerprints of SNMV RNA2 showed that one of the smaller oligonucleotides, denoted t21 (arrowed in Figure 1a) did not lie on this regular pattern and, moreover, had a slightly variable second dimension mobility relative to adjacent digestion products. No oligonucleotides were expected to appear in that position based on the known SNMV RNA2 sequence (Haseloff and Symons, 1982). In parallel experiments SNMV RNA2 was digested to mononucleotides with ribonuclease T2 and the sample was examined for the presence of unusual nucleotides in a two-dimensional t.l.c. system commonly used for tRNAs (Nishimura, 1979). The results (Figure 1b) showed a small amount of one other product (denoted X) besides the four major nucleotides. The fingerprints suggested, and the base analysis confirmed, that SNMV RNA2 contains an unusual nucleotide. An RNase T1 digestion product and a minor base of identical mobilities to those found in SNMV RNA2 were also seen in digests of VTMoV RNA2 (data not shown).

Of the possible unusual products of RNase T2 digestion which would appear at or near the position of X in the two-dimensional system shown in Figure 1b, most are ribonuclease-resistant dinucleotides (Nishimura, 1979). To see if this was true of X, a complete RNase T2 digest of SNMV RNA2 was fractionated by electrophoresis on DEAE paper at pH 3.5. (In this system oligonucleotides migrate more slowly than mononucleotides.) This experiment (Figure 1c) revealed a slowly migrating minor product. This product co-migrated with X in the first dimension solvent of the t.l.c. system, while X co-electrophoresed with the slowly migrating product on DEAE paper electrophoresis, showing that they were one and the same.

The electrophoretic mobility of X (which partly depends on the number of phosphate negative charges present) suggested that X might carry at least three, rather than two, phosphate groups, since its mobility was within the range expected for trinucleotides. Furthermore, a product with similar, though not identical chromatographic and electrophoretic mobilities [denoted P1X where a distinction from T2X is necessary (see Figures 2 and 4)] could be isolated from samples of SNMV RNA2 digested with nuclease P1 under conditions which are normally sufficient to cleave 3′-5′ phosphodiester bonds adjacent to sites of 2′ ribose methylation (Silberklang *et al.*, 1979). (RNase T2 is unable to cleave these bonds because it requires a free 2′ ribose hydroxyl to form an obligatory 2′,3′ cyclic phosphate intermediate, whereas nuclease P1, which is a sugar-unspecific nuclease, can cleave these bonds although at a reduced rate). These observations suggested that X was not a simple methylated dinucleotide. Another possibility for a slowly migrating digestion product on DEAE paper electrophoresis was a phosphorylated end group, but this was difficult to reconcile with the circularity of the virusoid molecule and was inconsistent with X's chromatographic mobility. A more attractive explanation was that X was a complex oligonucleotide produced by ligation of RNA2 to the circular form, perhaps containing the 2′ phosphomonoester, 3′-5′ phosphodiester structure described by Konarska *et al.* (1981), or the branched 2′-5′, 3′-′5 structure described by Wallace and Edmonds (1983) in digests of mammalian hnRNA. Therefore, the structure of X was further characterised, using material isolated on a preparative scale by DEAE paper electrophoresis.

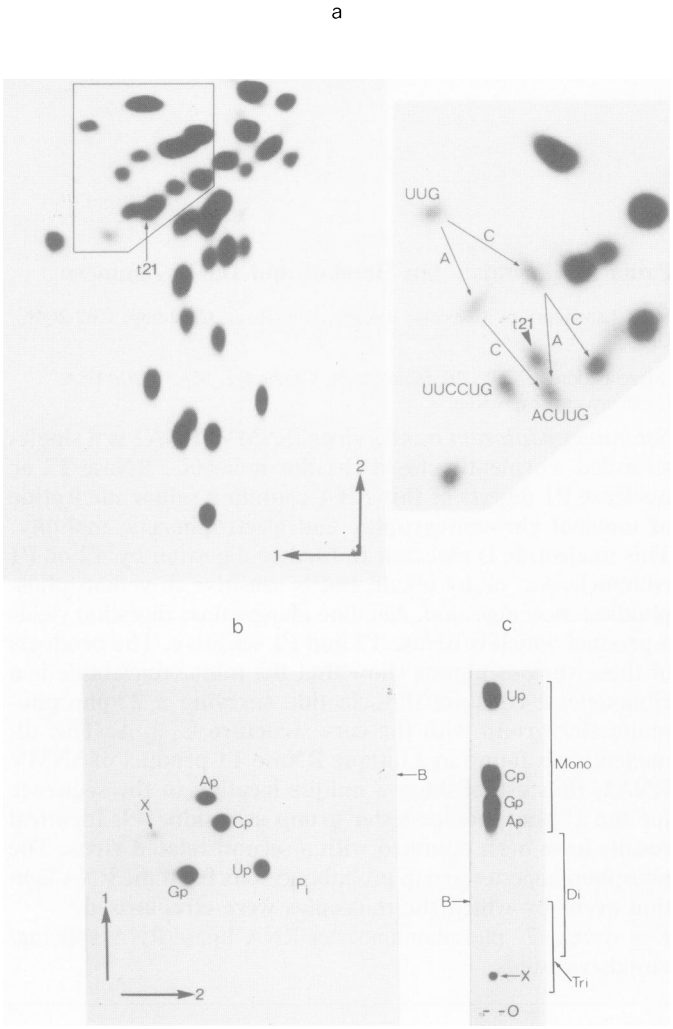

Fig. 1. (**a**) Fingerprint of [^{32}P]SNMV RNA2. First dimension: electrophoresis on cellulose acetate in pH 3.5 pyridine acetate containing 7 M urea and 2 mM EDTA. Second dimension: homochromatography using a 3%, 30 min hydrolysed homomix. An enlarged version of the area outlined on the complete fingerprint, but taken from a different experiment, is shown in the right hand panel to illustrate the variable second dimension mobility of t21. Graticule lines (mobility shifts from a UUG reference point) are marked on the inset – t21 runs off the graticule. (**b**) Two-dimensional cellulose t.l.c. analysis of the products of RNase T2 digestion of [^{32}P]SNMV RNA2. First dimension: isobutyric acid:0.5 N NH_4OH (5:3, v:v). Second dimension: isopropranol:conc. HCl:water (70:15:15, v:v:v). X denotes a product of unusual mobility and B the mobility of the blue marker dye xylene cyanol FF in the first dimension. (**c**) Electrophoresis of the products of RNase T2 digestion of [^{32}P]SNMV RNA2 on Whatman DE81 paper in pH 3.5 pyridine acetate buffer containing 2 mM EDTA. Range of mobilities of mono-, di- and tri-nucleotides tabulated by Barrell (1972) and van de Voorde *et al.* (1974) are marked. Abbrevations X and B as in (**b**).

Structure of X

Digestion of X with calf intestinal phosphatase yielded either two, or three products, depending on the conditions. The end products of digestion were inorganic phosphate, and a product migrating faster than all the mononucleotides on chromatography in solvent 1, suggesting that it contained more than one base per phosphate (Figure 2, lane a2). Partial phosphatase digestion yielded an additional third product migrating faster than X, but slower

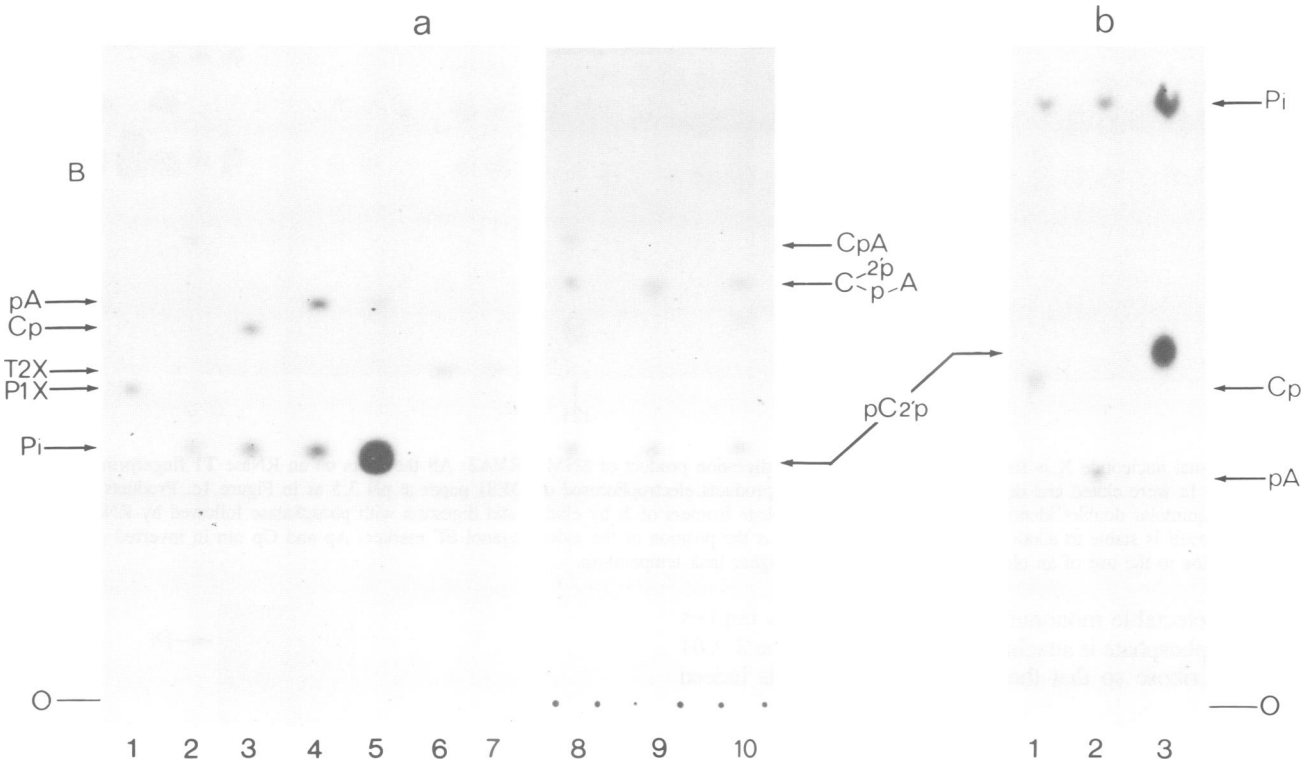

Fig. 2. Analysis of digestion products of spot X by cellulose t.l.c. in (**a**) first dimension solvent (isobutyric acid:0.5 M NH_4OH, 5:3) and (**b**) second dimension solvent (isopropanol:conc. HCl:water, 70:15:15). Samples are: **lane a1** P1X, undigested; **lane a2** P2X digested with 0.25 units CIP; **lane a3** as in **a2**, further digested with 1 unit RNase T2; **lane a4** as in **a2**, further digested with 1 μg nuclease P1; **lane a5** P1X digested with 1 μg VPDE; **lane a6** T2X undigested; **lane a7** as in **a6** after overnight incubation in 0.3 N NaOH at 37°C; **lane a8** T2X digested with 0.02 units CIP; **lane a9** as in **a8**, further digested with 1 μg nuclease P1; **lane a10** as in **a8**, further digested with 1 unit RNase T2. All redigestion volumes were 5 μl. **Lanes b1, b2** and **b3** are portions of the same samples shown in **lanes a3, a4** and **a5**. Mobilities of markers are shown on the extreme left and right of the figure. pA and Cp are the positions of unlabelled marker nucleotides included in samples **a1**–**7** and **b1**–**3**. Pi is the position of [^{32}P]inorganic phosphate run alongside **lanes a8**–**10**. Structures deduced for unknowns are shown between panels **a** and **b**. B indicates the mobility of xylene cyanol FF in the solvent system of **panel a**.

than the totally dephosphorylated oligonucleotide (Figure 2, lane a8). These observations suggested that X contained two phosphomonoester groups of differing susceptibility to phosphatase. Extensive digestion of either T2X or P1X with phosphatase, followed by digestion with RNases T2 or P1, yielded inorganic phosphate plus single products co-migrating with unlabelled marker Cp and pA respectively (Figure 2, lanes a3–4 and b1–2). In contrast similar treatment of partial phosphatase digests, while resulting in the appearance of Cp and pA and the disappearance of the fastest migrating product, gave no detectable digestion of the intermediate product (Figure 2, lanes a9 and 10). Since RNase T2 cleaves 3′-5′ phosphodiester bonds, and leaves a 3′ phosphate (Uchida and Egami, 1971), while RNase P1 cleaves only 3′-5′ phosphodiester bonds (Fujimoto *et al.*, 1974), and leaves a 5′ phosphate, these results define the core structure of X after phosphatase treatment as C3′p5′A, ruling out the presence of 2′,5′ phosphodiester bonds.

The fact that X was resistant to nuclease digestion before dephosphorylation, and sensitive afterwards, together with the observation that partial phosphatase digestion yielded an intermediate product which was still resistant to both nucleases T2 and P1 strongly suggested that X was a dinucleotide containing a 2′ phosphomonoester, 3′-5′ phosphodiester bond. The chromatographic mobility of T2X was consistent with that reported for $C^{2'p}_{3'p_{5'}}Ap$ (Kikuchi *et al.*, 1982). This would also explain its electrophoretic mobility (slower than a dinucleotide) and RNase resistance.

While such dinucleotides are resistant to all known endonucleases, they can be digested by the 3′ exonuclease venom phosphodiesterase (VPDE) (Konarska *et al.*, 1981). To see if this was true of X, a sample of P1X was digested with VPDE without prior dephosphorylation. Two main products were observed, one co-chromatographed with an unlabelled pA marker, while the other migrated with a mobility close to that of pC3′p in both dimensions of the chromatography system consistent with its being pC2′p (Figure 2, lanes a5 and b3). With increasing time of digestion, starting before digestion of X had gone to completion, the amount of pA present was reduced with the concomitant appearance of inorganic phosphate. We believe this is due to the activity of 5′ nucleotidase contaminating the VPDE rather than an intrinsic activity of the VPDE itself. With this reservation, the products of digestion are those expected of the structure (for P1X) $pC^{2'p}_{3'p_{5'}}A$. In particular, the release of pA shows that the phosphate not accounted for either as the 5′-terminal phosphate of a P1 digestion product or by the internal 3′-5′ phosphodiester bond must be attached to the 5′-terminal C residue of the dinucleotide, consistent with the appearance of a second product migrating like pC3′p. The results thus far, however, did not conclusively locate this extra phosphate. Conceivably, a base phosphorylation on the C residue, although unprecedented, might result in ribonuclease resistance before dephosphorylation. If this were true, however, the ribonuclease resistant dinucleotide should have a free 2′ ribose hydroxyl and therefore be sensitive to alkali. Figure 2 lane a7 shows that a sample of T2X which was treated with 0.3 M alkali for 18 h at 37°C co-chromatographed with an untreated control sample,

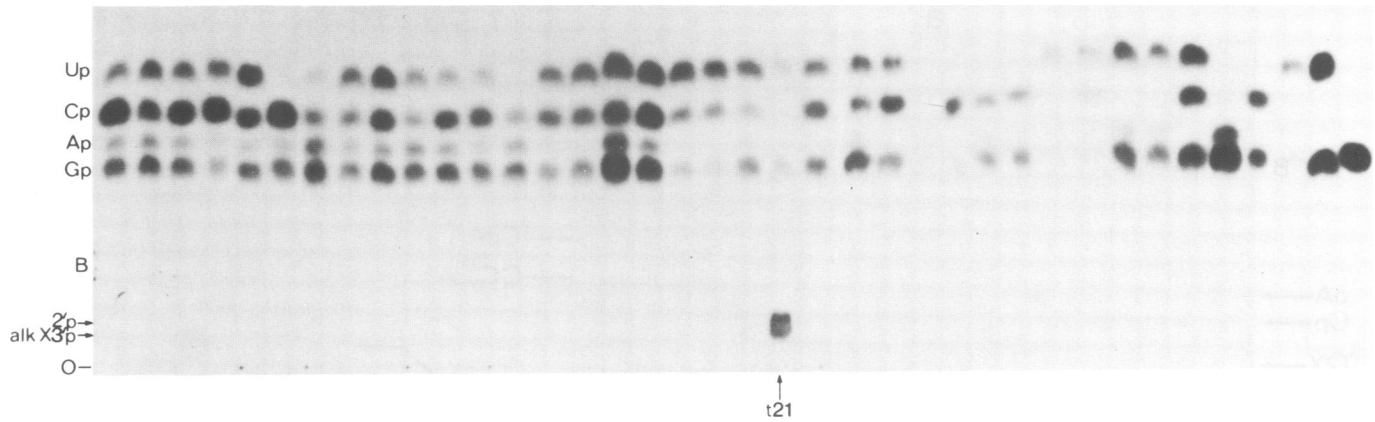

Fig. 3. The unusual nucleotide X is found in a unique RNase T1 digestion product of SNMV RNA2. All the spots on an RNase T1 fingerprint such as that shown in Figure 1a were eluted and digested with alkali, and the products electrophoresed on DE81 paper at pH 3.5 as in Figure 1c. Products of digestion of t21 yielded an equimolar doublet identified as the 2′ and 3′ phosphate isomers of X by elution and digestion with phosphatase followed by RNase T2 or nuclease P1. X itself is stable to alkali (see Figure 2, lane a7). B is the position of the xylene cyanol FF marker. Ap and Gp ran in inverted order compared with Figure 1c due to the use of an older batch of buffer and a higher tank temperature.

yielding no detectable mononucleotide products. This implies that the extra phosphate is attached to the C-residue at the 2′-OH group of the ribose so that the core structure of X is indeed $C^{2'p}_{3'p5'}A$.

Mapping the 2′ phosphomonoester

We next returned to the T1 RNase fingerprint of SNMV RNA2 to ask whether the anomalous migration of oligonucleotide t21 was due to its containing X, and if so whether this defined X's location in the SNMV RNA2 sequence. Figure 3 shows an experiment in which all the products of T1 RNase digestion of SNMV RNA2 were eluted from a fingerprint, such as that shown in Figure 1, digested to completion with alkali, and the products separated on DEAE paper by electrophoresis at pH 3.5. X was found among the digestion products of t21 and in no other oligonucleotide. The other products of alkaline digestion of t21 are Up and Gp, giving a base composition of (Up,$C^{2'p}_{3'p5'}$Ap)Gp which is consistent with the position of t21 on the fingerprint. Given this chain length, the molar yield of t21 was found to be 1.2 relative to other T1 products whose stoichiometry was known from the sequence. Together, these results suggest that X occurs at a unique site in the virusoid molecule, as expected for a ligation junction.

Since t21 yielded only two products of ribonuclease digestion besides Gp, comparison of P1 and T2 (or alkali) digests of t21 was sufficient to order them. Figure 4 shows that P1 digestion of t21 yields P1X and pG, together with inorganic phosphate released by the 3′ nucleotidase activity of nuclease P1. (From the specificity of RNase T1, G must be the 3′-terminal residue of t21 to which the 3′ phosphate was originally attached.) This implies that U is the 5′-terminal residue of t21 (since t21 is a product of T1 digestion the 5′-terminal residue carries no 5′ phosphate and is released as an unlabelled nucleoside in a P1 digest). Thus the sequence of t21 is (G)U$C^{2'p}_{3'p5'}$AGp.

The sequence GUCAG does not appear in the previously determined SNMV RNA2 sequence (Haseloff and Symons, 1982), but routine sequencing of cDNA clones of SNMV RNA2 that have since been constructed (J.Haseloff, unpublished data) revealed the presence of an extra C after residue 48 (Figure 5). The revised sequence beginning at residue 47 is GUCAG, which matches the sequence of t21. In retrospect, omission of the C from the original sequence at this position can be understood in the light of the 2′ phosphomonoester attached to it, since this would

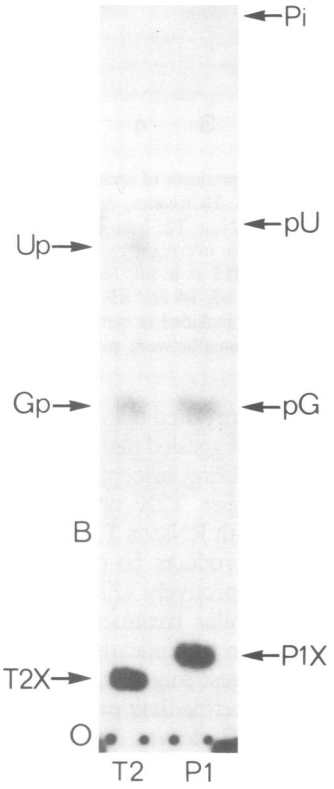

Fig. 4. Comparison of RNase T2 and P1 digestion products of t21 allows deduction of the sequence. Equal aliquots of a sample of t21 eluted from a fingerprint such as in Figure 1a were digested separately with RNase T2 and nuclease P1 and the products electrophoresed on DE81 paper at pH 3.5 as in Figure 1c. The presence of Up among the RNase T2 products and the absence of pU from the nuclease P1 products defines U as the 5′-terminal residue of t21 (see text).

prevent the enzymatic and alkaline cleavages which are the basis of the direct RNA sequencing method used to determine this part of the sequence. VTMoV RNA2 also has an extra C at the corresponding position when cDNA clones are examined (not shown). VTMoV RNA2 yields X on complete digestion with RNases T2 or P1, and a spot of identical mobility to t21 on an RNase T1 fingerprint. We conclude that the 2′ phosphomonoester

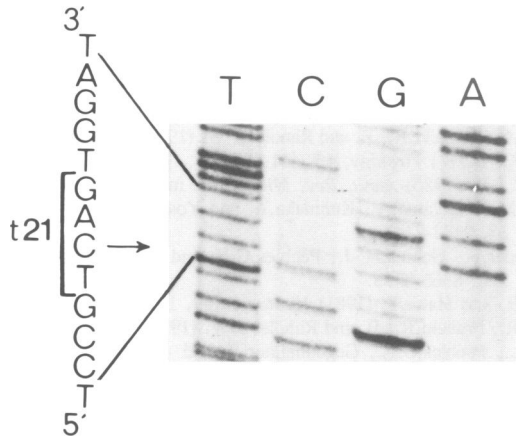

Fig. 5. Sequence of an SNMV RNA2 cDNA clone in M13mp11 reveals an extra C residue at position 49 (arrowed). Fractionation was on a 6% acrylamide 7 M urea salt gradient gel (Bankier and Barrell, 1983). The cloned insert is complementary to the packaged RNA2, hence the sequence read (shown on the left beginning at T44) is the plus strand. The sequence corresponding to RNase T1 digestion product t21 is bracketed.

in SNMV RNA2 is at residue 49 and that VTMoV RNA2 also has this modification at the same residue.

Discussion

The 2′ phosphomonoester probably marks a ligation site

2′ Phosphomonoester, 3′-5′ phosphodiester bonds are known to result from one class of RNA ligation reactions (Konarska *et al.*, 1981). Formation of the 2′ phosphomonoester during ligation results from a sequence of steps whose end result is the displacement of a 2′,3′ cyclic phosphate group at the 3′ end of an RNA by a 5′ phosphate group. Enzymes catalysing this type of reaction have been described in extracts of wheat germ, *Chlamydomonas*, and yeast. In the latter case the enzyme was identified by its activity in tRNA splicing, and no other substrates are known (Peebles *et al.*, 1979; Greer *et al.*, 1983). The wheat germ enzyme is probably also involved in tRNA splicing (Tyc *et al.*, 1983; Gegenheimer *et al.*, 1983), but has a wider substrate specificity – indeed it first came to light by virtue of its activity on a 73 residue RNase T1 fragment of tobacco mosaic virus RNA (Konarska *et al.*, 1981).

A structural characterisation alone cannot prove that virusoid RNAs are circularised by this plant ligase, since we cannot be certain that this is the only possible route to 2′ phosphomonoester synthesis. However, ligation *via* the mechanism used by this class of enzymes is the only precedent for the formation of a 2′ phosphomonoester in an RNA chain, and a ligation step is almost certainly required to circularise the virusoid RNA molecule. In all probability, the presence of this unusual bond in circular virusoid RNA reflects the ligation event by which the molecule was circularised *in vivo*, and marks the position of the ligation joint.

It is noteworthy that virusoid RNAs can be isolated carrying stoichiometric amounts of the 2′ phosphomonoester whereas in yeast tRNAs this residue is rapidly removed. It is not clear whether this represents a real difference between the two sytems, or simply the rapid protection of the 2′ phosphate group by packaging into virus particles.

Confirmation of a prediction made from sequence comparisons

Our conclusion that the 2′ phosphomonoester marks the site of ligation agrees with a prediction made by Symons *et al.* (1984) which was based on sequence comparisons. These workers suggested that three virusoid RNAs whose sequences they had determined (Haseloff and Symons, 1982; Haseloff, Davies and Symons, unpublished results) all contained a section of sequence homologous to the ends of a linear viral satellite RNA [satellite tobacco ringspot virus (Sat-TRSV) RNA (G.Bruening, personal communication)] previously thought to be unrelated to them. The ends of the linear molecule in their alignment coincide with the position at which we have found the 2′ phosphomonoester group in circular SNMV and VTMoV RNA2. Clearly, both results are consistent with the idea that this position is a site of ligation and are mutually confirmatory.

Do virusoids and viroids share a common ligation mechanism?

Virusoids and viroids are thought to have a number of similarities in both structure and replicative forms, so it would be of interest to know if their mechanisms of ligation were the same. In fact, a role for the plant 2′ phosphomonoester forming ligase in viroid circularisation has previously been proposed on the basis of *in vitro* experiments. These show that the wheat germ and *Chlamydomonas* enzymes are capable of circularising linear potato spindle tuber viroid (PSTV) RNA, whereas little or no circularisation was observed with yeast tRNA ligase or with bacteriophage T4 RNA ligase (Branch *et al.*, 1982; Kikuchi *et al.*, 1982).

These experiments show that the 2′,3′ cyclic phosphate termini that are a unique requirement for ligation by this class of enzymes are present in the population of linear viroid RNA, but ligation at a unique site *in vitro* has not yet been demonstrated. Tyc *et al.* (1983) have noted in the context of tRNA ligation that it is difficult to rule out the possibility that some proportion of *in vitro* ligations by the wheat germ enzyme may be due to repair of nicked molecules because of that enzyme's wide substrate specificity. However, Kikuchi *et al.* (1982) were able to show by polynucleotide kinase labelling that their preparations of linear potato spindle tuber viroid (PSTV) RNA had 5′ ends mapping at two major sites. Both these sites, like the ligation site in SNMV and VTMoV virusoid RNAs, are at CpA bonds, but it is not yet known whether either or both are used for PSTV RNA ligation *in vivo*.

Implications for the overall mechanism of virusoid biosynthesis

The only known ligation mechanism leading to 2′ phosphomonoester formation requires a 2′,3′ cyclic phosphate end on the linear precursor of the circular molecule, which in turn would require post-transcriptional synthesis, consistent with a cleavage and ligation mechanism for virusoid biosynthesis overall.

Viroid RNAs were the first circular RNAs to be discovered (Sanger *et al.*, 1976; McClements and Kaesberg, 1977). Circularisation of the excised intron has since emerged as a general theme of many splicing pathways. Since the discovery of splicing possibility of shared pathways of cleavage and ligation between viroids (and the more recently discovered virusoids) and cellular introns has been pointed out by a number of authors both in general and more specific terms. Our results are consistent with the idea that virusoid RNA circularisation uses a ligation step resembling that implicated in the pathway of plant tRNA splicing.

Materials and methods

Viruses

Solanum nodiflorum mottle virus (from an inoculum provided by Olwen Stone) and velvet tobacco mottle virus (from an inoculum provided by Richard Francki) were grown in *Nicotiana clevelandii* (authorised by licences PHF 241/103

and PHF 241/104 respectively from the Ministry of Agriculture, Fisheries and Food, London) and purified as previously described (Kiberstis and Zimmern, 1984).

Protoplasts

Isolation and culture of *N. clevelandii* protoplasts was as described (Kiberstis and Zimmern, 1984) except that KH_2PO_4 was omitted from the culture medium (Aoki and Takebe, 1969) and the pH was adjusted to 6.5 with either KOH or MES (2[N-morpholino]ethane sulphonic acid). Carrier-free [^{32}P]orthophosphate (Amersham) was added to a final concentration of 0.1 – 1.0 mCi/ml (depending on the experiment), 5 – 6 h after resuspension of the protoplast pellet in PEG at the start of inoculation. Protoplasts were harvested after 20 – 27 h of incubation.

RNA extraction and purification

Freshly harvested protoplasts were resuspended at ~5 x 10^5/ml in 50 mM Tris Cl (pH 7.5), 0.2 M NaCl, 5 mM EDTA, lysed with 1% SDS and extracted with phenol or in 0.1 M NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, lysed with 2% SDS and extracted with phenol-chloroform (no significant differences were observed between the two procedures). The aqueous phase was ethanol precipitated, washed with ethanol, dried briefly, resuspended in formamide or urea dye buffer and loaded on either 6% or 3% acrylamide 7 M urea 40 cm thin gels run in Tris-borate-EDTA buffer (TBE) pH 8.3 (Sanger and Coulson, 1978; Bankier and Barrell, 1983). The RNA2 band (identified by its co-migration with marker RNA2 from virions) was cut out and the RNA eluted by shaking in 0.5 M ammonium acetate, 1% SDS and 1 mM EDTA for 5 – 6 h at room temperature (0.25 ml/slice). Eluted RNA was precipitated with ethanol, resuspended in 0.3 M potassium acetate, re-precipitated, and used as below.

RNA fingerprinting

Standard fingerprinting methods of Sanger and co-workers including elution and redigestion conditions, were used as described by Barrell (1972). 2 mM EDTA was added to all electrophoresis buffers to prevent streaking of highly phosphorylated nucleotides.

Base analysis

RNA2 was digested with either: (i) a mixture of 50 units/ml RNase T2 (Sankyo) 0.25 mg/ml pancreatic RNase/0.25 mg/ml, T_1 RNase in 50 mM ammonium acetate buffer, pH 4.5, 2 mM EDTA at an enzyme to substrate ratio of 1 μl mixed enzymes/4 μg carrier yeast tRNA or (ii) 1 mg/ml nuclease P1 (Sigma) in 50 mM ammonium acetate buffer, pH 5.2 at 1 μg P1/4 μg RNA. Digestion was for 3 h at 37°C in both cases. 1 – 5 μl digests were loaded onto 20 x 20 cm cellulose t.l.c. plates (E.Merck, Darmstadt) and chromatographed in the solvents described by Nishimura (1979). 5 – 10 μl digests were electrophoresed on Whatman DE81 paper at pH 3.5 as described by Barrell (1972). All the separations shown in the figures were done on old Whatman DE81 paper, where mobilities are as given in the reference. Current Whatman DE81 is a slightly altered formulation and mobilities will therefore be different. U.v. markers were from Sigma and P-L Biochemicals.

Further characterisation of spot X

X was eluted from DE81 paper with triethylamine bicarbonate and digested with 0.2 mg/ml VPDE (Worthington) as described (Barrell, 1972). Redigestions with calf intestinal phosphatase (Boehringer), followed by RNase T2 and nuclease P1 digestion were as described by Kikuchi *et al.* (1982) except where noted in the figure legends.

Construction and characterisation of SNMV and VTMoV RNA2 clones

Clone construction (covered by MAFF licence no. PHF 241/105 and GMAG licence no. D 607/11/80) will be described elsewhere. Standard M13 sequence reactions were run as described (Bankier and Barrell, 1983).

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Preliminary results show additional modifications on other virusoid RNA species that may be related to the ligation process, and suggest that the mechanism of 2′ phosphomonoester synthesis in virusoid RNA is more complex than (though probably related to) that in tRNA.