CONVERSION OF CIRCULAR VIROID MOLECULES TO LINEAR STRANDS

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1. Introduction

We have shown that viroids are single-stranded covalently-closed RNA molecules with mol. wt ~120 000. In their native state they exist as largely double-stranded rod-like structures which are characterized by a serial arrangement of helical segments and internal single-stranded loops [1–3]. In our viroid preparations we find usually <1% linear molecules which were assumed to arise through 'nicking' of viroid circles during the isolation procedure [1].

For certain studies the availability of large quantities of linear viroid molecules would be of great advantage. It has been long established that bi- and trivalent metal cations catalyze the cleavage of RNA even at very low concentrations [4–6]. Thus at slightly alkaline pH Mg^{2+} cleave RNA molecules generating 5′-hydroxyl end groups and 2′- and 3′-phosphates, a reaction which has successfully been used to specifically cleave individual tRNA species [7]. We have applied this Mg^{2+}-catalyzed phosphodiester cleavage to different viroids and found that one single nick occurred under a wide range of controlled conditions. This cleavage produces linear viroid molecules of the same size as the covalently-closed RNA circles from which they originate. In contrast to the circles, these linearized viroids are susceptible to enzymatic 5′-phosphorylation and exhibit lower thermal stability. From these findings it appears that linear viroid molecules are produced inadvertantly if Mg^{2+}-containing buffers are used during viroid purification.

Abbreviations: $PSTV_{tom}$, potato spindle tuber viroid from tomato (cv. Rentita); CEV_{gyn}, citrus exocortis viroid from *Gynura aurantiaca;* $CPFV_{tom}$, cucumber pale fruit viroid from tomato (cv. Rentita); $ChSV_{cin}$, chrysanthemum stunt viroid from *Cineraria cruentus* (cv. Hansa); $ChCMV_{chr}$, chrysanthemum chlorotic mottle viroid from *Chrysanthemum morifolium* (cv. Deep Ridge)

Nomenclature: 5′-polynucleotide kinase from T4 phage-infected *E. coli,* EC 2.7.1.78

2. Materials and methods

2.1. *Electrophoretic analysis of the kinetics of Mg^{2+}-catalyzed nicking of circular viroid (PSTV) RNA molecules on 5% polyacrylamide gels*

Highly purified viroid (PSTV) RNA (100 μg) were subjected to 5 mM Mg^{2+} in 500 μl 25 mM glycine–NaOH buffer (pH 9.0) at 37°C. Aliquots (25 μg) were taken at the indicated time intervals, mixed with 50 μl 50 mM sodium EDTA (pH 8.3) and immediately frozen in dry ice to terminate the reaction. For electrophoresis the samples were thawed at 4°C, made to 8 M urea and 10 μg RNA were analyzed together with an untreated aliquot in slots of a 5% polyacrylamide (5% acrylamide, 0.125% bisacrylamide) slab gel (23 × 26 cm, 3 mm thick) under fully denaturing conditions as deduced from melting analysis [8]. The gel contained 8 M urea in buffer (pH 8.3) of low ionic strength (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM sodium EDTA). The electrode buffer was of the same low ionic strength, but without urea. Electrophoresis was carried out for 6 h at 200 V and 30 mA in a 37°C room resulting in

an actual gel temperature of 43–45°C. Under these conditions xylene cyanole FF marker dye migrates about twice the distance of the band of linear viroid molecules. The gel was stained for 30 min in 1% methylene blue dissolved in 15% acetic acid and destaining was carried out in 15% acetic acid with several changes of the destaining solution.

2.2. *Electrophoretic analysis of a viroid (PSTV) preparation after Mg^{2+}-catalyzed nicking followed by 5′-^{32}P end-group labelling and autoradiography*

Highly purified viroid (PSTV) RNA (50 μg) were subjected for 60 min at 37°C to 5 mM Mg^{2+} in 250 μl 25 mM glycine–NaOH buffer (pH 9.0). The reaction was terminated by mixing the sample with 50 μl 50 mM sodium EDTA (pH 8.3) followed by immediate freezing in dry ice. 5′-^{32}P end-group labelling of the sample was carried out with T4-induced phosphokinase as in [2]. Aliquots of 5 μg Mg^{2+}-treated viroid RNA were made to 8 M urea and analyzed together with an untreated 5 μg control sample on a 5% polyacrylamide (5% acrylamide, 0.125% bisacrylamide) slab gel (23 × 26 cm, 3 mm thick) under non-denaturing (a) and fully denaturing (b) conditions as deduced from melting analysis [7].

(a) Non-denaturing conditions: 8 M urea in buffer (pH 8.3) of high ionic strength (180 mM Tris, 180 mM boric acid, 5 mM sodium EDTA) at 10–12°C gel temperature as obtained by running the electrophoresis in a cold room at 6–7°C. The electrode buffer was of the same ionic strength, but without urea. Gel run was for 18 h at 250 V and 130 mA. Xylene cyanole FF used as marker dye migrates ~1 cm ahead of the viroid band under these conditions.

(b) Fully denaturing conditions were exactly as in section 2.1.

The bands in both gels were made visible by staining with methylene blue as in section 2.1. In order to localize the fraction of linearized and 5′-^{32}P-labelled viroid molecules as originating from Mg^{2+}-catalyzed cleavage of viroid circles, the gels were autoradiographed on Kodak-RP/S X-Omat X-ray film.

2.3. *Differentiated melting curves of PSTV*

All experiments were carried out in 0.019 M sodium-cacodylate, 1 mM EDTA (pH 6.8) using a microcuvette containing 60 μl sample volume [8].

3. Results and discussion

The five viroid species $PSTV_{tom}$, CEV_{gyn}, $CPFV_{tom}$, $ChSV_{cin}$ and $ChCMV_{chr}$ were propagated and purified as in [1,9]. It should be emphasized that

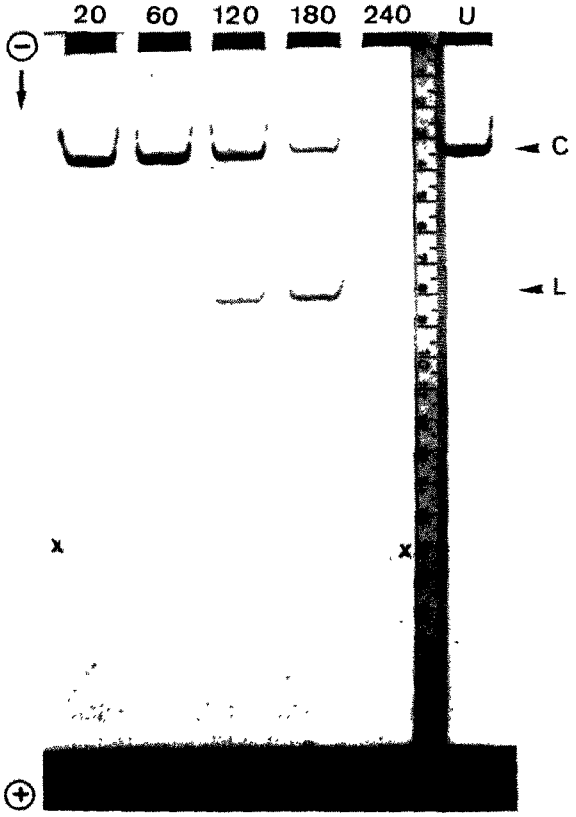

Fig.1. Electrophoretic analysis of the kinetics of Mg^{2+}-catalyzed nicking of circular viroid (PSTV) RNA molecules on 5% polyacrylamide gels: C, circular viroid molecules; L, linear viroid molecules; F, viroid fragments; X, xylene cyanole FF marker dye; U, untreated control sample in 25 mM glycine-NaOH buffer without Mg^{2+}. After 180 min Mg^{2+}-treatment, nicking at multiple sites occurs as indicated by a faint and diffuse staining of the polydisperse viroid fragments arising. After 240 min the bands of both circular and linear forms have largely disappeared, whereas bands of viroid fragments appear in the 10% 'barrier'-gel at the bottom of the gel slab. The numbers above the slots give the time of Mg^{2+} treatment at 37°C.

these and all our earlier preparations were always purified in the presence of an excess of EDTA to complex any polyvalent cations. The purity and individuality of these preparations were routinely checked by fingerprinting as in [10]. Our viroid preparations are ~100% circular molecules as demonstrated by the absence of 3′- and 5′-end groups [1] and by their homogeneous melting curves [1,3,8].

Preparations of circular viroids were systematically incubated in slightly alkaline Mg^{2+}-containing buffers under a variety of conditions. Figure 1 shows the kinetics of the Mg^{2+}-induced alteration of PSTV as analyzed on a 5% polyacrylamide gel under strictly denaturing conditions. A new and faster migrating band of linear viroid molecules appears as demonstrated by 5′-end group labelling and further electrophoretic analysis (fig.2). The intensity of the band of linear molecules increases with the length of time during which Mg^{2+} is present, whereas the band of circular molecules decreases concomitantly.

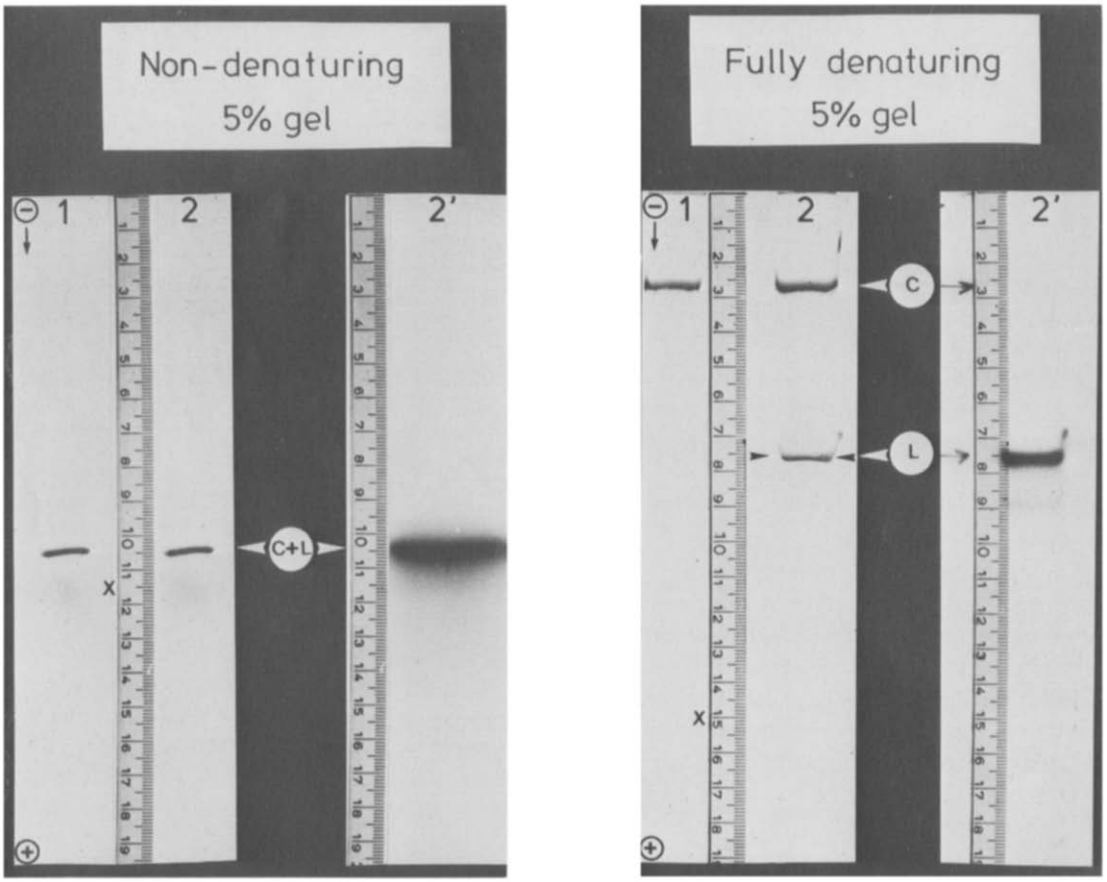

Fig.2. Electrophoretic analysis of a viroid (PSTV) preparation after Mg^{2+}-catalyzed nicking followed by 5′-^{32}P end group labelling and autoradiography: C, circular viroid molecules; L, linear viroid molecules; X, xylene cyanol marker dye. Slots 1, untreated control samples; slots 2, Mg^{2+}-treated viroid RNA; slots 2′, autoradiographs corresponding to slots 2 of the stained gels, respectively. Comparison between the pattern of the stained gel and the corresponding autoradiograph shows that under non-denaturing conditions (a) the viroid circles and the Mg^{2+}-nicked and 5′-^{32}P end-group-labelled viroid molecules co-migrate. In contrast, under fully denaturing conditions (b) the Mg^{2+}-nicked 5′-^{32}P-labelled linearized molecules migrate almost three times as fast as the unfolded viroid circles which are not susceptible to 5′-end group labelling. Upon longer exposure of the autoradiographs additional faint bands of a series of smaller viroid fragments can be observed which evidently originate from cleavage at multiple sites.

Further analysis of the cleavage kinetics showed that ~50% of the viroid circles are converted into linear forms after about 25 min, 50 min, 150 min and 360 min at 60°C, 50°C, 37°C and 25°C, respectively. In all kinetic experiments additional faint bands of faster-migrating material become detectable after longer incubation periods. These bands contain distinct viroid fragments evidently generated by additional nicking at multiple sites. It should be pointed out that all 5 viroid species tested showed practically the same susceptibility to Mg^{2+}-catalyzed cleavage. This observation is in good agreement with their very similar structural features as reflected in their nearly identical thermodynamic and kinetic behaviour [3].

The fact, that only the nicked viroid molecules are susceptible to 5′-^{32}P end-group labelling allowed a more detailed electrophoretic analysis.

Mg^{2+}-nicked and 5′-end group labelled viroid preparations were separated together with a non-treated sample under non-denaturing and fully denaturing conditions and the patterns of the stained gels were compared with the corresponding autoradiographs (fig.2). It is clearly shown that Mg^{2+} produce one single nick per viroid molecule thus converting viroid circles to linear forms. Under non-denaturing conditions the stained circular and the nicked and labelled linear molecules comigrate in 5% gels which shows that the secondary structure of both forms must be very similar. This finding is not surprising because under non-denaturing conditions a single nick would certainly not change the rod-like secondary structure of the native viroid molecule significantly because of its high stability [2,3,10]. We wish to point out that in our routine procedure for viroid purification all preparative electrophoretic runs are carried out under non-denaturing conditions in 5% gels, where both intact and nicked circular viroid molecules comigrate. Therefore, we can exclude the possibility that we are selecting for viroid circles and thus inadvertantly losing linear viroid molecules.

On the other hand, circular and linear viroid molecules can be well separated on the same 5% gel under fully denaturing conditions as provided by low ionic strength buffer and 8 M urea at elevated gel temperature (fig.1,2). Under such conditions the linear molecules are migrating almost 3-times as fast as the covalently-closed but unfolded viroid circles. It has been observed that the linear forms of single-stranded SV40 and ΦX174 DNA also migrate faster than the corresponding circular forms [11]. It is assumed that unfolded circles are physically retarded in their migration, whereas linear molecules seem to thread their way 'end on' through the interstices of the polyacrylamide gel matrix [12].

For 5′-end group analysis the band of the 5′-labelled linear molecules of PSTV was recovered from the polyacrylamide gel by electrophoretic elution [13] and precipitated together with 50 μg carrier tRNA. An aliquot of this material was completely digested to the mononucleotides with nuclease P_1 [14]. This digest was fractionated by cellulose thin-layer chromatography as in [14], the 5′-^{32}P nucleotides were located by autoradiography and recovered for Cerenkov counting. The ratio of 5′-terminal pA, pC, pU and pG was 38 : 17 : 25 : 20, respectively, indicating only a low degree of specificity of this Mg^{2+}-catalyzed cleavage.

The nicking of viroids may be detected clearly also in thermodynamic experiments. Beside the well-known differentiated melting curve of pure circular viroids [3,8] the corresponding curves of isolated linear forms and of a mixture of both forms are depicted in fig.3. The midpoint-temperature and the cooperativity of the main denaturation process of the

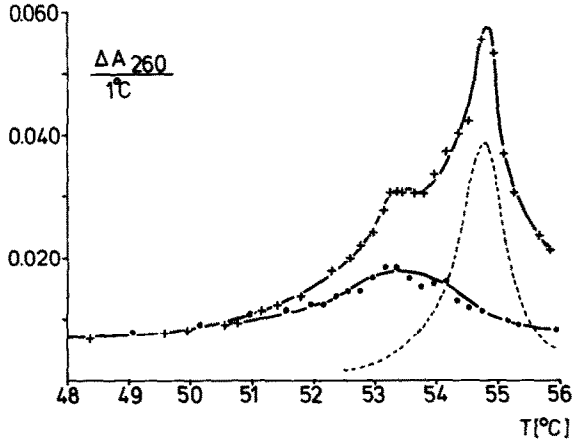

Fig.3. Differentiated melting curves of PSTV. (○) Isolated nicked forms, A_{260} (20°C) = 0.9; (- - -) isolated circular form according to [11], A_{260} (20°C) = 0.35; (+) mixture of nicked (A_{260} = 0.9) and circular (A_{260} = 0.35) forms.

linear forms are significantly lower than those of the circular form. These results confirm our earlier theoretical prediction [3] and support our interpretation of two melting peaks which appeared after repetitive heating in our previous calorimetric studies [15]. From a close inspection of the present melting curve of the linear forms which shows a considerable amount of melting even at 48°C it may be concluded that not only one species with a reduced cooperativity contributes to the experimental curve but that a number of different transitions arising from heterogeneously nicked molecules are superimposed. This result is in good agreement with the end group analysis.

In bioassays using dilution end-point titration on a molar basis we found that for instance in the case of PSTV ~10% of the tomato plants became infected, when they were inoculated with a solution containing 10^5-10^6 linear molecules/plant. This result is in a marked contrast to the $\sim10^3-10^4$-times higher infectivity of the isolated viroid circles [1]. Comparable results were obtained with the other 4 viroid species tested. There are several explanations possible. First, the specific infectivity of more or less all linear molecules may be $\gtrsim10^3$-times lower than the one of viroid circles. In the other extreme, only after a nick at a very specific site, full infectivity might be retained, whereas all other linear molecules are completely non-infectious. It is also conceivable that nicked viroid molecules are far more sensitive to enzymatic degradation which would lead to their inactivation during the inoculation process. Finally, we cannot exclude that all linear molecules are principally non-infectious, so that the comparatively low infectivity of our samples would be attributed to a contamination with viroid circles of $<0.1-0.01\%$. Recircularization of linears to fully infective circles may be inhibited in vivo, because RNA ligase needs 3′-OH and 5′-phosphate end groups. It is interesting to note that viroid circles were obtained again when inocula from individual symptom-bearing plants at the end-point dilution from the bioassay of isolated linear molecules were used for further propagation.

Our results clearly demonstrate that Mg^{2+} generate linear viroid molecules by introducing one single nick into viroid circles. Linear viroid molecules have been described as a major product of viroid replication [16,17]. According to the routine purification procedure used in these studies [18–20] the corresponding viroid preparations were subjected for longer periods to 3 mM Mg^{2+} in alkaline buffers (pH 9.0) at elevated temperatures. Consequently, the relatively high and varying percentage (30–80%) of linear viroid molecules obtained can be attributed to Mg^{2+}-catalyzed cleavage of viroid circles. In fact, following this purification procedure [18–20] we also obtained 40–70% linear forms and a correspondingly lower yield of viroid circles. It is evident, therefore, that all traces of free polyvalent cations must be excluded during viroid purification if reliable conclusions concerning the occurrence of linear viroid molecules in vivo are to be drawn. On the other hand, it is not possible at present to decide whether the few linear molecules in our completely Mg^{2+}-free preparations are due to the action of nucleases or whether they are actual intermediates of viroid replication.

Acknowledgements

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