FURTHER CHARACTERIZATION OF THE DISCONTINUITIES IN CAULIFLOWER MOSAIC VIRUS DNA

K. E. RICHARDS, H. GUILLEY and G. JONARD

Laboratoire de Virologie, Institut de Biologie Moléculaire et Cellulaire du CNRS, 15 rue Descartes, 67000 Strasbourg, France

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1. Introduction

The DNA molecule composing the genome of cauliflower mosaic virus (CaMV) is double-stranded and circular. Strand separation experiments show, however, that the two complementary DNA chains composing the circle are not covalently closed but are interrupted at well-defined sites, twice in one strand and once in the other [1–4]. The single break (Δ1) in the so-called α-strand, the strand which is transcribed, is taken as the zero point of the conventional restriction map [5]; the two breaks in the complementary β-strand, Δ3 and Δ2, are situated at 0.20 and 0.53 map units, respectively. CaMV DNA from which the discontinuities have been eliminated by cloning in pBR322 is fully infectious but the DNA isolated from the progeny of such infection is once again interrupted [6,7], suggesting that the breaks are essential for completion of the infectivity cycle and that a precise mechanism exists for introducing them into the viral DNA.

Originally, the discontinuities were thought to be either nicks or gaps [8], but sequence analysis [9] revealed that, at least for Δ2 and Δ3, the 3′- and 5′-extremities of the interrupted strand overlap, by 18 residues in the case of Δ2 and by at least 2 residues for Δ3. Here, we show that Δ1 also has an overlapping sequence and that the 3′-extremities of all 3 overlapping structures are heterogeneous in length.

2. Materials and methods

2.1. *Cauliflower mosaic virus DNA*

CaMV (isolate Cabb B-S) was propagated in turnips (*Brassica rapa* var. Just right) and purified as in [10]. Viral DNA was extracted from the purified virus according to [5].

Cloned DNA consisting of viral DNA inserted into the *Sal*I site of pBR322 was kindly provided by Dr Hohn.

2.2. *Restriction of the DNA and 5′-end-labelling of the resulting fragments*

Digestions of the DNA with restriction enzymes *Bgl*II, *Hin*fI and *Hin*dIII (BioLabs.) were performed as recommended by the supplier. Details of dephosphorylation and 5′-end-labelling of the DNA fragments are in [9].

2.3. *Polyacrylamide gel electrophoresis and recovery of the labelled DNA fragments*

5′-End-labelled DNA fragments were fractionated by electrophoresis onto 8% 2 mm thick polyacrylamide gels using the buffer system at pH 8.3 [11]. After autoradiography the desired bands were excised from the gel. The DNA was eluted by agitation of the crushed gel band overnight at 37°C in 1 ml of 500 mM NaCl, 50 mM Tris–HCl (pH 7.9) and filtration of the slurry through a syringe equipped with a piece of filter paper at its extremity. Finally, the DNA fragments were precipitated with ethanol in the presence of 10 μg carrier tRNA.

2.4. *Strand separation of DNA fragments*

The 5′-end-labelled DNA fragments were subjected to strand separation by heating at 90°C for 2–3 min in 50 μl 30% DMSO, 1 mM EDTA. The samples were then rapidly chilled on ice and immediately loaded onto a 6% 2 mm thick polyacrylamide slab gel [12].

2.5. *Sequencing reactions*

Single-stranded 5′-end-labelled DNA fragments were partially degraded using the base-specific chemical cleavage reactions of Maxam and Gilbert [13].

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The cleavage product was fractionated on 8% 0.5 mm thick polyacrylamide sequencing gels [14].

3. Results and discussion

In [9] several isolated short 5′-^{32}P-labelled double-stranded restriction fragments were isolated, each encompassing 1 of the 3 discontinuities. Denaturation of such fragments yield three 5′-end-labelled single-stranded species, the two smaller fragments corresponding to the two segments of the interrupted strand and the longest fragment to the continuous complementary strand. Sequence determination along the uninterrupted strand defines the sequence across the discontinuity while analysis of the fragment having its 5′-end at the discontinuity fixes its 5′-limit with respect to the continuous complementary strand [9]. The approximate 3′-limit of each discontinuity was also determined by reading sequence in from a 5′-labelled restriction site toward the 3′-end of the discontinuity [9]. These results show that such analysis underestimates the 3′-extent of the interrupted strand by 10–12 nucleotides because sequencing gel usually cannot be read to its end.

The 3′-termini of the discontinuities can be labelled by incubation with terminal deoxynucleotidyl transferase and [α-^{32}P]TTP [2] but we have encountered difficulties in using this technique to introduce enough label at the discontinuities for sequencing purposes ([9] and other observations). Furthermore, single-stranded fragments ending in a discontinuity and which have been 3′-^{32}P-labelled in this fashion often migrate as rather broad bands in polyacrylamide gels. Sequence gels prepared from such material generally gave sequence ladders in which the basic pattern of bands was obscured by 'echos', with the basic pattern repeated several times but out-of-phase by one or more nucleotides.

In [9] we established that the discontinuities are homogenous at their 5′-extremities but the above observations suggest that the 3′-termini may be heterogenous in length. To test this idea, we have precisely sized short single-stranded fragments 3′-terminating at each discontinuity in DNA sequencing gels. Fig.1 schematizes the 5′-^{32}P-labelled restriction fragments used as starting material. The fragments were chosen so that in no case do >100–150 nucleotides separate the interruption from the restriction site having the discontinuous strand as 5′-terminus. The corresponding 5′-end-labelled fragments were also isolated from cloned CaMV DNA [5], which lacks the discontinuities. Each fragment was denatured and the single strands separated by electrophoresis through a polyacrylamide gel (fig.2). The 5′-terminal sequence of each fragment was determined to identify those species of interest. In the case of Δ1 and Δ2, the strand containing the discontinuity and having the restriction site as 5′-terminus and the interruption as 3′-terminus (strand I in fig.1) migrated as one rather broad band whereas strand I of Δ3 migrated as a polydisperse set of bands (fig.2).

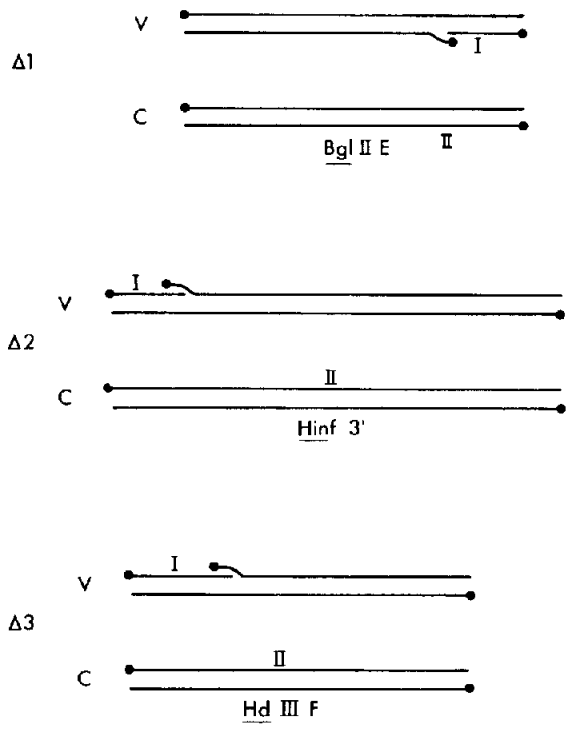

Fig.1. Structure of restriction fragments used to characterize the discontinuities. Restriction fragments were obtained from CaMV Cabb B-S virion DNA (V) or from Cabb B-S DNA cloned in the *Sal*I site of pBR322 (C) [5]. The location of the fragments in the CaMV sequence [9] is as follows: *Bgl*II E, 7664–7692, *Hin*f 3′, 4111–4664; *Hin*dIII F, 1514–1950. The 5′-extremities of the DNA strands are indicated by (•).

To determine the precise length of the interrupted strands an aliquot of each strand I from fig.2 was electrophoresed in denaturing conditions through a DNA-sequencing gel along with a sequence ladder prepared from the corresponding fragment from the

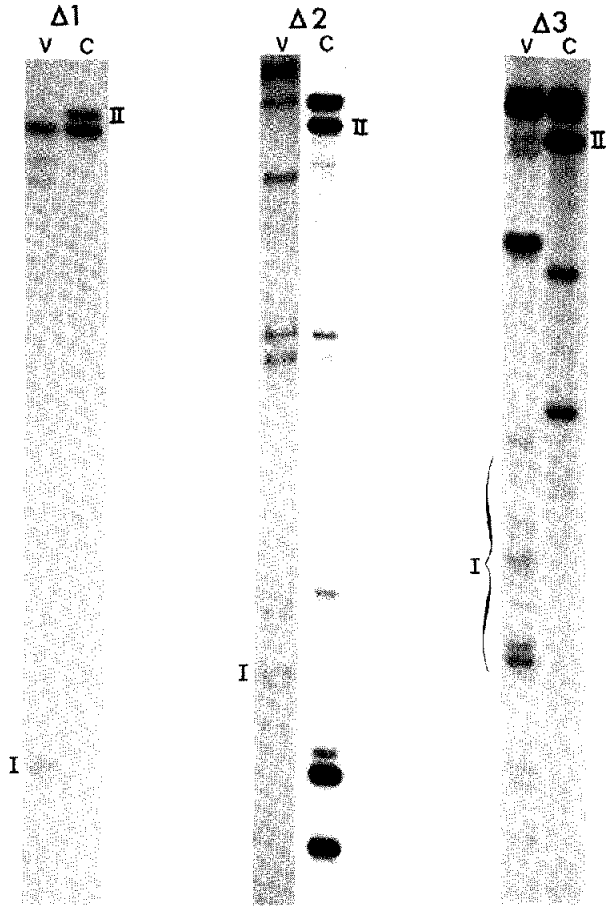

Fig.2. Strand separation of 5′-^{32}P-end-labelled restriction fragments from virion (V) and cloned (C) CaMV DNA in a 6% polyacrylamide gel. The identity of each restriction fragment is given in fig.1. The bands indicated by I were shown by sequence analysis to correspond to the strand with 5′-terminus at the restriction site and 3′-terminus at the interruption (see fig.1) while bands indicated by II are the corresponding strand from non-interrupted (cloned) DNA. The slowly migrating pair of fragments in Δ2 V is the two strands of *Hin*f fragment 3 (positions 6164–6753) which contaminate fragment 3′ while the bands migrating about half-way down the gel are double-stranded DNA fragments, presumably arising from incomplete denaturation of the starting material. The pair of slowly migrating bands in Δ3 V and C contain the 2 strands of the contaminating *Hin*dIII E (5376–5851) plus the non-interrupted strand of *Hin*dIII F. The two most rapidly migrating bands in Δ3 C probably arise from single-strand nicked molecules of *Hin*dIII E.

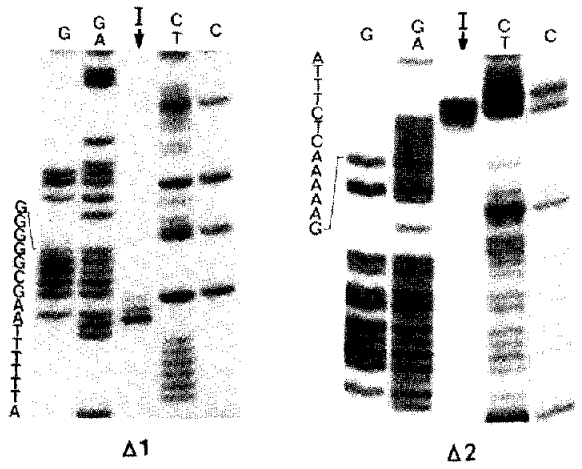

Fig.3. Autoradiograms of 8% polyacrylamide–urea gels showing sequence ladders from the 5′-^{32}P-labelled strands from cloned DNA which traverse Δ1 and Δ2 (strand II in fig.1,2). In each case the middle lane contains the 5′-^{32}P-labelled interrupted fragment from virion DNA (strand I in fig.1,2) with 3′-extremity at the discontinuity.

cloned DNA (strand II). It can be seen from the autoradiogram of the gel (fig.3) that strand I from Δ1 is heterogenous in length, containing 3 species differing by one nucleotide. Since all 3 species have the same 5′-terminus (the restriction site) it follows that the length heterogeneity must be at the 3′-end. Thus for Δ1, the extent of overlap with respect to the 5′-terminus of the discontinuity determined [9] is 8, 9 and 10 nucleotides (fig.4). Similar analysis reveals that strand I from Δ2 likewise consists of 3 discrete species corresponding to an overlap of 17, 18 and 19 residues (fig.4). The second of these 3 extremities was detected by direct sequence analysis of 3′-end-labelled material [9]. Finally, the 3′-extremity of Δ3 proved to be extremely polydisperse: strands with 3′-termini at positions 1643–1646 were predominant (fig.4) but minor species ending at positions 1656–1657, 1666–1669, 1674–1675 were also detected.

The overlapping structures at the discontinuities of CaMV DNA are almost certainly generated in the course of DNA replication but their role in the viral infection cycle remains unclear. The 3′-terminal length heterogeneity presumably arises from imprecise termination of the replicative event although the possibility that it results from nucleolitic degradation of longer overlapping structures cannot be exluded.

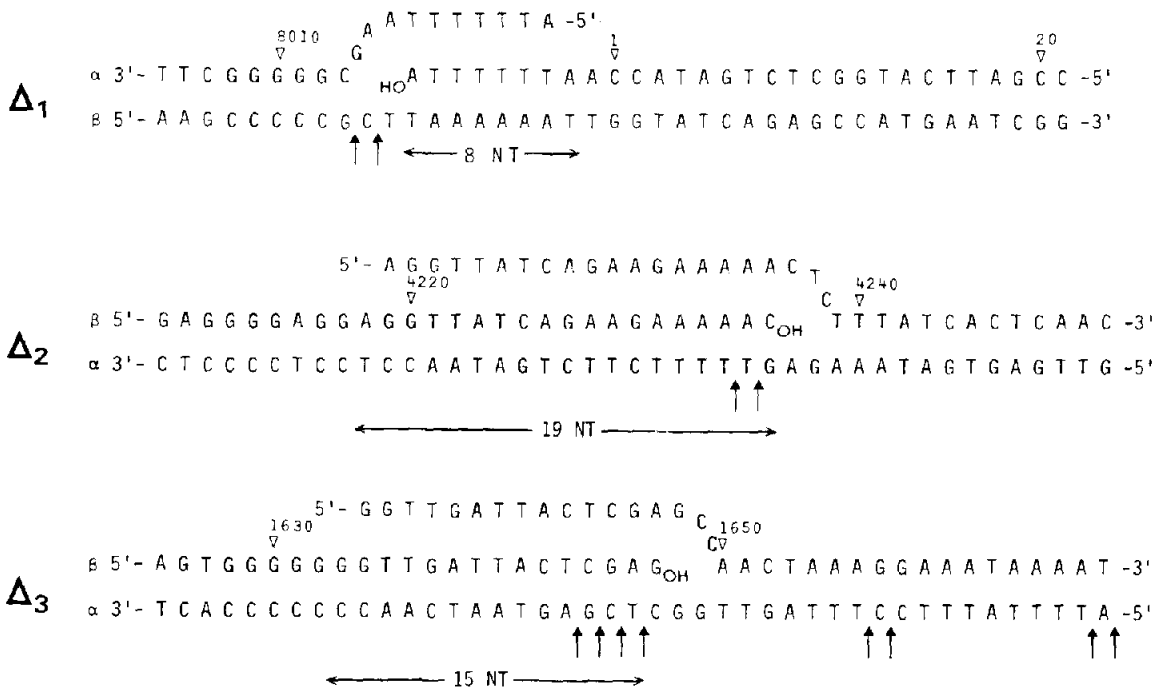

Fig.4. Sequence around the discontinuities of CaMV Cabb B-S DNA. The location of the 5'-extremity of the discontinuities and the sequence of the complementary strand is from [9]. Arrows indicate the positions of other 3'-termini detected.

Acknowledgements

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