A putative primer for the replication of cauliflower mosaic virus by reverse transcription is virion-associated

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We have isolated, from a cauliflower mosaic virus (CaMV) preparation, an RNA species (75 nucleotides) covalently linked to the 5'-end of the CaMV DNA fragment (sa-DNA) which is complementary to the CaMV 35 S RNA transcript 5'-end. Site-specific cleavage located a unique m^7G residue in the RNA moiety of sa-DNA suggesting that it is host cell $tRNA_i^{met}$. sa-DNA is reminiscent of retrovirus 'strong-stop' DNA and is evidence for the reverse transcription model of CaMV replication.

DNA plant virus *CaMV* *$tRNA^{met}$* *Reverse transcription*

1. INTRODUCTION

Recent observations [1–4] have revealed striking similarities between cauliflower mosaic virus (CaMV) and animal retroviruses (review [9]). These include the discovery of potential priming sites for both plus- and minus-strand synthesis, by the reverse transcription mode, of the circular double-stranded 8 kilobase (kb) DNA found encapsidated in virions. The priming sites are located at fixed positions adjacent to 3 single-strand discontinuities (G1 in the minus-strand, G2 and G3 in the plus-strand) in virion DNA.

A 14 nucleotide sequence of perfect homology with the 3'-end of plant $tRNA_i^{met}$ [5,6] is present in the DNA minus-strand adjacent to G1 [2–4]. An 8.2 kb polyadenylated RNA (35 S RNA), which has a terminal directly-repeated sequence of 180 nucleotides [7,8], is transcribed from the minus-strand such that the sequence complementary to $tRNA_i^{met}$ is positioned some 600 nucleotides from the 35 S RNA 5'-terminus.

It has been proposed [3,4,9] that replication is initiated by a host cell $tRNA_i^{met}$ which binds to its complementary sequence in 35 S RNA and that reverse transcription proceeds to the RNA 5'-end to produce a molecule analogous to retrovirus 'strong-stop' DNA (see [10]).

We have previously detected [1], in CaMV-infected cells, a small minus-stranded DNA molecule (sa-DNA) covalently linked to a polyribonucleotide tract. This molecule maps between the origin of 35 S RNA transcription and G1 in virion DNA and thus could represent an early reverse transcript. This view is supported by findings reported here.

2. EXPERIMENTAL

2.1. *Virus and nucleic acid isolation*

CaMV (isolated Cabb B-JI) was propagated in turnip (*Brassica rapa* cv. Just Right). Virus was isolated from leaves (4 weeks post-inoculation) as in [12], except that the sucrose gradient ultracentrifugation step was omitted. The final virion pellet was resuspended in 100 mM Tris–HCl (pH 7.4), 2.5 mM $MgCl_2$ and incubated with DNase 1 (10 μg/ml) at 37°C for 10 min. Virions were then disrupted by digestion with pronase (0.5 mg/ml) in 10 mM EDTA, 1% sodium dodecyl sulphate at 65°C for 10 min. Nucleic acid was extracted from disrupted virions and from CaMV-infected turnip leaves as in [1].

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2.2. *5′-end-labelling*

Fifty μg of CaMV virion DNA was electrophoresed through a 1.5% agarose slab gel and stained with ethidium bromide (1 μg/ml). A gel slice containing sa-DNA was crushed and incubated overnight at 37°C in 0.2 M sodium acetate (pH 6.0). Eluted nucleic acid was concentrated by ethanol-precipitation. Nucleic acid was dephosphorylated with bacterial alkaline phosphatase (BRL) (100 units) in 100 mM Tris-HCl (pH 8.0), 120 mM NaCl at 65°C for 60 min. Dephosphorylated 5′-termini were labelled with [γ-^{32}P]ATP (1 kCi/mmol) in 60 mM Tris-HCl (pH 7.8), 15 mM dithiothreitol, 10 mM $MgCl_2$, 330 nM ATP using 5 units of T_4 polynucleotide kinase (BRL) at 37°C for 30 min.

2.3. *m^7G cleavage*

Site-specific cleavage of RNA at m^7G [11] was as follows: nucleic acid in 20 μl of 0.2 mM EDTA was mixed with 20 μl of 0.1 M NaOH and incubated at 20°C for 15 min. pH was adjusted to 5 with 2 μl of 1 M acetic acid followed by addition of 40 μl of 0.3 M aniline hydrochloride in 0.2 M sodium acetate (pH 4.5); incubation was at 37°C for 4 h.

2.4. *Gel electrophoresis*

Electrophoresis of DNA and RNA, denatured by glyoxylation as in [1], was in 1.2% agarose or 2.5% (or 3.5%) low-gelling temperature (LGT)/0.5% agarose composite gels in 25 mM Tris-acetate (pH 7.9), 1 mM EDTA, 5 mM sodium acetate buffer or in 25 mM Tris-phosphate (pH 7.6), 2 mM EDTA. CaMV-specific sequences were detected by blot-hybridisation using CaMV DNA, ^{32}P-labelled by nick-translation, to probe nitrocellulose transfers. 5′-end-labelled fragments were separated on denaturing thin (0.3 mm) 6% polyacrylamide gels at 25 W constant power [13]. Radioactivity was detected by autoradiography with intensifying screens at −70°C.

3. RESULTS

In [1] we reported that the CaMV-minus-strand small DNA fragment sa-DNA was present in both the cellular DNA and RNA fractions from infected turnip leaves but was not detectable in preparations of virion DNA. By omitting the sucrose gradient step in the virion isolation method [12], DNA extracted from DNase 1-treated virions contains a DNA species (fig.1a) which co-migrates in LGT agarose with sa-DNA found in cellular RNA (fig.1d). These molecules were degraded by subsequent DNase treatment (fig.1b,e) and were also sensitive to alkali hydrolysis (fig.1c,f) consistent with the loss of an RNA fragment [1]. A second species, sb-DNA [1] was also virion-associated (fig.1a).

To establish the polarity of the RNA fragment, we purified sa-DNA by excising from an agarose gel a fraction of virion DNA which contained it (fig.1g). Re-electrophoresis and detection by blot-hybridisation showed that a significant enrichment of sa-DNA had been achieved (fig.1h,i). The recovered sa-DNA was 5′-labelled in vitro with [γ-^{32}P]ATP and polynucleotide kinase and electrophoresed on a 6% polyacrylamide thin gel. From several runs, we estimated the size of sa-DNA as 680 nucleotides (fig.2a,3). Following DNase treatment of 5′-end-labelled sa-DNA, the 680 nucleotide species was degraded and an additional 5′-labelled molecule of 75 nucleotides, presumably RNA, was observed (fig.2b). From

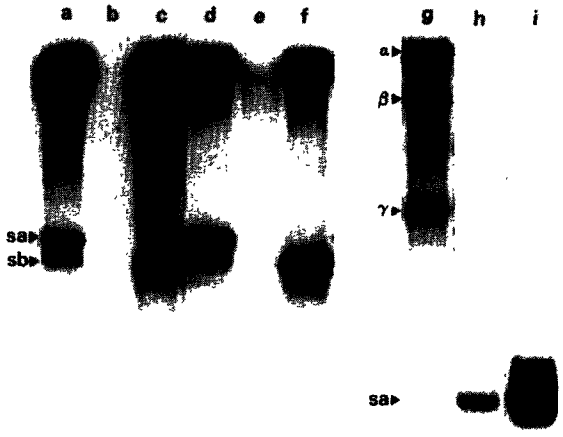

Fig. 1. Detection of sa-DNA by blot-hybridisation in virion DNA (a–c) and cellular RNA (d–f) after electrophoresis in 2.5% LGT agarose. DNase 1 (b,e)- and alkali (c,f)-treated samples. Purified sa-DNA (h, overexposed in i) from virion DNA (g) run in 1.2% agarose. Sizes of CaMV DNA α, β and γ strands are 8 kb, 5.4 kb and 2.6 kb, respectively.

Fig. 2. (a) 5′-end-labelled sa-DNA, (b) treated with DNase 1 electrophoresed on a 6% polyacrylamide gel. XC, xylene cyanol. Sizes (nucleotides, nt) are 5′-end-labelled pAT153/Xho II fragments.

this, we conclude that sa-DNA consists of about 600 nucleotides of DNA covalently linked, at the DNA 5′-end, to 75 nucleotides of RNA.

Our contention is that the RNA in sa-DNA is host cell $tRNA_i^{met}$. Inspection of the nucleotide sequences of $tRNA_i^{met}$ from both bean [5] and wheat germ [6] reveals the presence of a unique m^7G residue located 45 nucleotides from the tRNA 5′-end. If the m^7G residue found in plant $tRNA_i^{met}$ is similarly placed in the putative $tRNA_i^{met}$ of sa-DNA, then site-specific cleavage at m^7G should result in an increase in mobility of sa-DNA due to the loss of 45 nucleotides. When sa-DNA present in cellular RNA (fig.3a) and that purified from

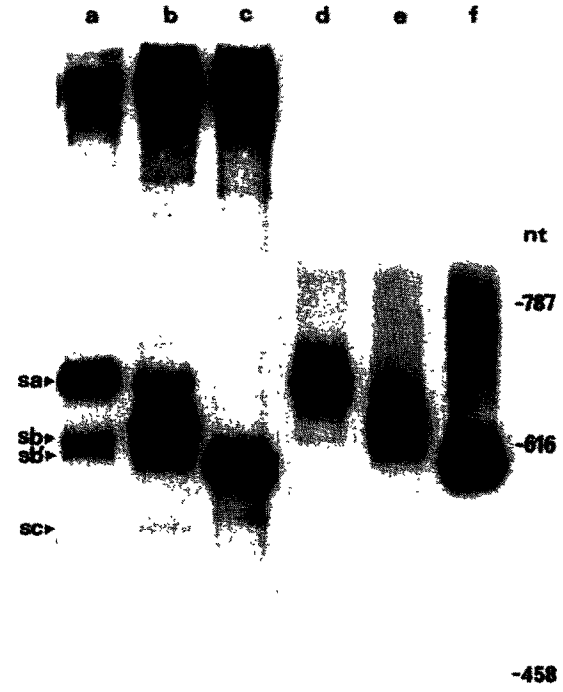

Fig. 3. Treatment of intact sa-DNA (a,d) in cellular RNA (a–c) and purified from virion DNA (d–f) with aniline hydrochloride to locate m^7G (b,e) and then hydrolysed with alkali (c,f) run on 3.5% LGT agarose. Size markers indicated were CaMV DNA *Eco*R1/*Sal*1 fragments.

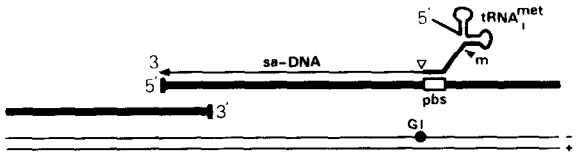

Fig. 4. Proposed structure of sa-DNA aligned with CaMV 35 S RNA (thick lines) and virion DNA near the minus-strand discontinuity G1. pbs, sequence complementary with $tRNA_i^{met}$; m, m^7G residue; (▽), DNA/tRNA junction.

virion DNA (fig.3d) was subjected to chemical cleavage specific for m^7G [11], its mobility in 3.5% LGT agarose increased exactly as predicted, consistent with removal of 45 ribonucleotides at the 5′-end (fig.3b,e). Subsequent alkali treatment hydrolysed the remaining 5′-terminal 30 ribonucleotides (fig.3c,f). It is interesting to note that sb-DNA in cellular RNA was resolved by LGT agarose into two components, b (615 nucleotides) and b′ (605 nucleotides) (fig.3a), whereas only one component was present in virion DNA (fig.3d, and not shown). The plus-strand fragment sc-DNA was described in [1].

4. DISCUSSION

We have further characterised an unusual CaMV DNA fragment, sa-DNA and demonstrate that a 75 nucleotide RNA species is covalently linked to its 5′-terminus. Site-specific chemical cleavage located a unique m^7G residue some 45 nucleotides from the 5′-end of the RNA moiety of sa-DNA. A m^7G residue is similarly located in $tRNA_i^{met}$ of both bean [5] and wheat germ [6]. The nucleotide sequence of $tRNA_i^{met}$ from turnip has not yet been determined but it is reasonable to suggest that it is similar to that from bean and wheat which in these distantly related species the sequence of $tRNA_i^{met}$ is virtually identical.

Few plant cytoplasmic tRNAs have been sequenced and we cannot totally discount the possibility that a different turnip tRNA species has an m^7G residue located in the same position as that in $tRNA_i^{met}$. However, since the 5′-end of the minus-strand polarity sa-DNA maps close to the CaMV virion DNA minus-strand discontinuity G1 [1], which itself is immediately adjacent to the 14 nucleotide CaMV DNA sequence perfectly homologous to the 3′-end of plant $tRNA_i^{met}$ [1–6], we believe this sufficient evidence to suggest that sa-DNA is a fragment of CaMV DNA, the 5′-end of which is covalently linked to the 3′-end of turnip $tRNA_i^{met}$.

We have not yet precisely mapped the termini of the DNA moiety of sa-DNA. However, a virion-associated molecule, closely resembling sa-DNA, called α_1, has been described [2]. The 3′-end of α_1 is coterminal with the 5′-end of 35 S RNA and its 5′-end, with a single terminal ribonucleotide, is located precisely at G1 [2]. It is likely that α_1 is sa-DNA which has lost most of the 5′-tRNA and is thus similar to the CaMV DNA fragment, sb-DNA, reported by us in [1].

Our proposed structure for sa-DNA as it relates to 35 S RNA and virion DNA is shown in fig. 4. This is the first evidence of a tRNA covalently-linked to plant DNA. The structure we propose for sa-DNA is directly analogous to minus-strand 'strong-stop' DNA of retroviruses (see [10]) and furnishes strong support for the model of CaMV DNA replication [3,4,9] by reverse transcription of 35 S RNA.

We do not yet understand the significance of this putative replication intermediate being virion-associated, although it is present in cellular DNA and RNA fractions as well [1]. Encapsidation could be fortuitous; alternatively, this may indicate an early function in the virus replication cycle.

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