SYNTHESIS OF DNA TRANSCRIPTS OF POTATO SPINDLE TUBER VIROID

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1. Introduction

Sequences in the DNA of several uninfected solanaceous host species have recently been shown to represent at least 60% of potato spindle tuber viroid (PSTV) [1]. PSTV replication is known to be inhibited by actinomycin D, which suggests involvement of DNA [2,3]. No new DNA sequences related to PSTV were found at detectable levels after infection of tomato plants with PSTV [1], which suggests that PSTV may be replicated on a DNA template that is already present in the uninfected host plant.

RNA-Directed DNA polymerase (reverse transcriptase) from avian myeloblastosis virus (AMV) lacks template specificity [4], making it useful for synthesizing DNA complements from a wide variety of RNA species. The RNA template to be copied must contain a natural double-stranded primer region or a region to which a complementary sequence can be hybridized, i.e., a poly(A) stretch [5,6]. DNA polymerase I from *Escherichia coli* is also reported to be able to synthesize complementary copies from a variety of natural heteropolymeric RNAs [7–10]. These complementary DNAs (cDNAs) have been used as hybridization probes for detecting and quantitating nucleic acid sequences.

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Thus, cDNA copies of PSTV could be used as sensitive probes to study relationships among viroids and to study possible structural similarities between viroids and their host genomes.

In this paper, we describe conditions for the synthesis of DNA transcripts of PSTV utilizing DNA polymerase I from *E. coli* and partially characterize the product. In addition, we report that PSTV does not possess poly(A) or poly(C) stretches in its structure, thus precluding the use of oncorna viral reverse transcriptase for cDNA synthesis.

2. Materials and methods

2.1. *Materials*

[^{3}H]-Labelled deoxynucleoside-5′-triphosphates were purchased from Amersham-Searle Co.*, Arlington Heights, IL, and from Schwarz-Mann Co., Orangeburg, NY. ^{125}I-Labelled dCTP was prepared as described [11]. Unlabelled deoxynucleoside-5′-triphosphates, $(rA)_n \cdot (dT)_{10}$, $(dT)_{10}$, and $(dG)_{12-18}$, were obtained from P.L. Biochemicals, Milwaukee WI. Actinomycin D was obtained from Calbiochem Co., La Jolla, CA. Electrophoretically pure pancreatic RNAase A was obtained from Worthington Biochemical Corporation, Freehold, NJ. *Aspergillus* S_1 nuclease was a gift of Dr Howard M. Temin.

PSTV was purified from tissues of PSTV-infected tomatoes as described [12,13]. Plant viral nucleic

acids were kindly supplied by Drs H. Fraenkel-Conrat, M. Zaitlin, I. R. Schneider, H. Lot, and J. M. Kaper. Qβ phage RNA and transfer RNA of *E. coli* were purchased from Miles Laboratories, Elkhart, IN. Samples of ^{3}H-labelled, singlestranded, short DNA chains of SV 40 were obtained from Dr P. K. Qasba. DNA-Calf thymus was a product of Calbiochem Co. CF-11 Cellulose and GF/C filters were obtained from Whatman, Inc., Clifton, NJ. All chemicals were of reagent grade.

Homogeneous preparations of *E. coli* DNA polymerase I were the generous gift of Dr L. Loeb. Reverse transcriptase was isolated from AMV and was purified using poly(rC)–agarose chromatography [14].

2.2. *Methods*

2.2.1. Reverse transcriptase assays

Reactions were carried out as described by Marcus et al. [14]. Assays containing $(rA)_n \cdot (dT)_{10}$ template-primer were used as our positive controls.

2.2.2. *E. coli* DNA polymerase I assays

Standard assays were carried out in 0.1 ml vol. buffer containing 50 mM Tris–HCl, pH 7.8, 5 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 0.5 mM $MnCl_2$, 50 mM KCl, 0.04% bovine serum albumin, 100 μM each of unlabelled dATP, dCTP, and dTTP, 40 μM of ^{3}H-labelled dGTP (spec. act. 500 cpm/pmol), 5 μg of actinomycin D, 30 ng *E. coli* DNA polymerase I and 2 μg of PSTV. Incubation was performed at 37°C for 30–60 min. Reactions were terminated with 5% trichloroacetic acid –0.01 M sodium pyrophosphate, and trichloroacetic acid-insoluble material was collected onto GF/C filters. Filters were dried and placed in vials containing 5 ml of toluene-based PPO–POPOP scintillation fluid and the radioactivity was determined in a Searle Mark III liquid scintillation spectrometer.

2.2.3. Analysis of DNA

For isolation of radioactive product from the reaction mixtures, assays were terminated by the addition of EDTA to a final concentration of 10 mM. Isolation and purification of the DNA product on CF-11 cellulose column were done as described [15].

Samples of ^{125}I-labelled DNA transcripts of PSTV, ^{3}H-labelled DNA of SV 40 and unlabelled PSTV markers were analyzed in 2.4% polyacrylamide gels for 45 min at 5 mA/gel as described previously [16]. After electrophoresis, the position of the DNA transcripts of PSTV and markers in gels was determined [13].

For determination of the specificity of DNA transcripts of PSTV, ^{125}I-labelled DNA (500 cpm) and several RNA species were incubated for 65 h at 41°C in 0.2 ml reaction mixtures containing 0.015 M Tris–HCl, pH 7.3, 0.15 M NaCl, 5×10^{-4} M EDTA, 0.1% sodium dodecyl sulfate, activated calf thymus DNA (1 μg/ml) and 38% formamide, as described by Beneveniste and Scolnick [17]. Hybrid yield was scored as the percentage of ^{125}I-labelled DNA recovered as S_1 nuclease-resistant, trichloroacetic acid-precipitable radioactive material [17].

3. Results

3.1. *Reaction of PSTV with reverse transcriptase*

Attempts to synthesize DNA transcripts of PSTV using AMV reverse transcriptase were unsuccessful. In all experiments with PSTV and d[^{3}H]TTP in the presence or absence of $(dT)_{10}$ or $(dG)_{12-18}$ primer, the incorporation of radioactive material into trichloroacetic acid-insoluble product was about 1% or less than that into the positive control (data not shown). Moreover, addition of unlabelled dATP, dCTP, and dGTP did not stimulate d[^{3}H]TMP incorporation. Similar results were obtained when d[^{3}H]GTP replaced d[^{3}H]TTP as the labelled substrate. Because we could not produce DNA transcripts of PSTV using the oncorna viral reverse transcriptase, the *E. coli* DNA polymerase I system was investigated.

3.2. *DNA synthesis with* E. coli *DNA polymerase I and PSTV*

The response of *E. coli* DNA polymerase I to PSTV template was tested by following the incorporation of d[^{3}H]GMP into acid-precipitable material. Synthesis of heteropolymeric DNA on PSTV template in the absence of added primer was observed (table 1). Annealling PSTV to $(dT)_{10}$ or $(dG)_{12-18}$ primer did not stimulate d[^{3}H]GMP incorporation. The reaction was dependent on the presence of all four deoxyribonucleoside triphosphates. The heteropolymeric reaction was inhibited by RNAase A but was relatively

Table 1
Template activity of PSTV with *E. coli* DNA polymerase I

Template-primer	Substrate	d[^{3}H]NMP incorporated (pmol/μg enzyme)
PSTV	^{3}H-Labelled dGTP, dATP, dCTP, dTTP	57.34
PSTV (Act. D, 50 μg/ml)	^{3}H-Labelled dGTP, dATP, dCTP, dTTP	51.55
PSTV + $(dT)_{10}$	^{3}H-Labelled dGTP, dATP, dCTP, dTTP	59.87
PSTV + $(dG)_{12-18}$	^{3}H-Labelled dGTP, dATP, dCTP, dTTP	58.23
PSTV	^{3}H-Labelled dGTP, dATP, dTTP	0.03
PSTV (RNAase, 10 μg/ml)	^{3}H-Labelled dGTP, dATP, dCTP, dTTP	5.99
PSTV + $(dT)_{10}$	d[^{3}H]TTP	0.03
PSTV + $(dG)_{12-18}$	d[^{3}H]GTP	0.04

Assays were carried out as described in Materials and methods. Incubation at 37°C for 30 min. Incorporation in the absence of added template-primer was substracted from all determinations.

insensitive to actinomycin D, indicating that PSTV itself was the template for the polymerase. Furthermore, DNA synthesis was not observed when d[^{3}H]TTP was used as the sole precursor in the presence of PSTV and $(dT)_{10}$ primer, indicating that poly(A) stretches are lacking [18] in the PSTV structure. Poly(C) stretches are also absent in the viroid structure because DNA synthesis was not observed when d[^{3}H]GTP was used as the sole precursor in the presence of PSTV and $(dG)_{12-18}$ primer. For 5 μg PSTV or less d[^{3}H]GMP incorporation was directly proportional to the concentration of the PSTV template and the kinetic of the reaction was linear up to 45 min (data not shown).

[^{125}I]-Labelled DNA of PSTV apparently contained a single product with a molecular weight approximately 5×10^4 (fig.1). The specificity of the DNA product was tested by hybridization of DNA

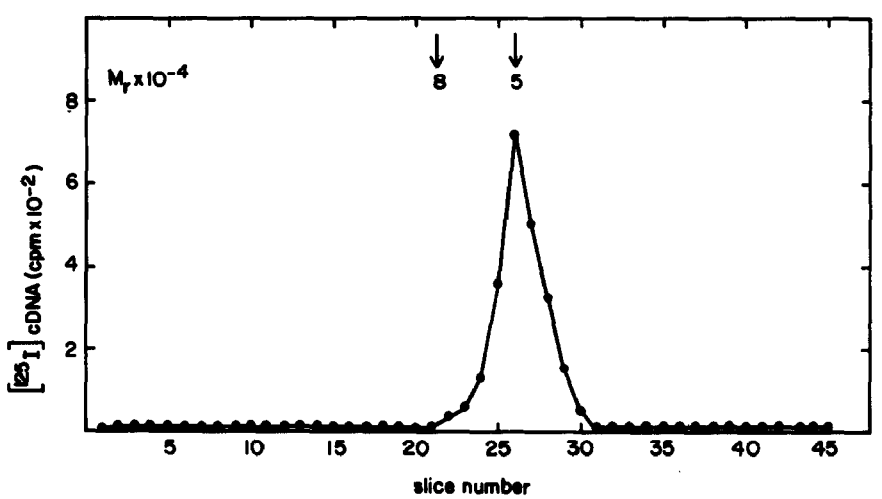

Fig.1. Gel electrophoretic pattern of pure ^{125}I-labelled DNA transcripts of PSTV electrophoresed in 2.4% polyacrylamide gel for 45 min at 5 mA/gel. Pure unlabelled PSTV (mol. wt 8×10^4) and ^{3}H-labelled single-stranded, short DNA chains of SV 40 (mol. wt approx. 5×10^4 [19]) were used as internal markers; arrows indicate the migration position of these markers.

Table 2
RNA specificity of PSTV-cDNA

RNA	% cDNA hybridized
Potato spindle tuber viroid	51
Brome mosaic virus	14
Cucumber mosaic virus	19
Tobacco mosaic virus	13
Turnip yellow mosaic virus	21
Tobacco ringspot virus	18
Satellite of tobacco ringspot virus	45
Qβ Phage	11
E. coli, transfer	9
No added RNA	9

DNA was synthesized with ^{125}I-labelled dCTP as the precursor utilizing PSTV as template. The product was isolated on CF-11 cellulose column as described in Materials and methods. Hybridization was in formamide as described in Materials and methods. Hybrids were scored by resistance to S_1 nuclease at 37°C for 1.5 h. Hybridization values are expressed as the percentage of ^{125}I-labelled cDNA of PSTV recovered as S_1 resistant, TCA-precipitable radioactive material. The results shown represent an average of three determinations.

to eight viral RNAs, as well as to *E. coli* tRNA. Hybrids formed between PSTV and DNA were much more resistant to S_1 nuclease treatment (51%) than the other hybrids (9–21%), with the exception of those formed with the RNA of the satellite of tobacco ringspot virus (45%) (table 2). The DNA contains sequences that cannot be degraded by S_1 nuclease (9% resistance with no added RNA). This may result from DNA sequences that have inherited secondary structure form the PSTV template or self annealling of the DNA product.

4. Discussion

The data presented show that PSTV can serve as template for DNA synthesis catalyzed by *E. coli* DNA polymerase I. The lack of template activity of PSTV in the AMV reverse transcriptase system is probably due to the absence of poly(A) or poly(C) stretches in the PSTV structure which could bind to an oligo(dT) or oligo(dG) primer, respectively. The lack of poly(A) sequences in PSTV structure is similar to observations with citrus exocortis viroid (CEV) because CEV was unable to hybridize with poly(U) [20]. Since synthesis of cDNA of PSTV takes place in the absence of a primer, an initiator site(s) for DNA synthesis may occur in *E. coli* DNA polymerase I. The endonuclease activity known to be associated with the enzyme could be a factor in the initiation of DNA synthesis. As *E. coli* DNA polymerase I can synthesize DNA complementary to PSTV in vitro, the DNA complementary to PSTV detected in normal solanaceous plants [1] could be synthesized by a host DNA polymerase enzyme(s) functioning under appropriate conditions as an RNA-directed DNA-synthesizing enzyme similar in function to the bacterial enzyme.

The size of the heteropolymeric DNA transcript of PSTV is 5×10^4 daltons, which is about half that of PSTV. When DNA was hybridized to PSTV, about 51% of the DNA hybridized. This incomplete hybridization could result from some sequences being more easily copied than others or from part of the DNA not being complementary to PSTV. That the sequences of nucleotides in the DNA product are complementary to PSTV and not just randomly ordered was demonstrated by hybridization of DNA to several RNA species. Maximum hybrid yield was obtained with DNA–PSTV duplexes, indicating that the nucleotide sequence of the DNA is at least partially complementary to PSTV. Our hybridization results also indicated the presence of some common nucleotide sequences in PSTV and the RNA of the satellite of tobacco ringspot virus (SAT–TRSV). It is worth noting that the two RNA species have similar molecular weights [12,21] and that the DNA of one host species of SAT–TRSV (*Phaseolus vulgaris* L. cv. Black Valentine) contains sequences complementary to PSTV [1].

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