SYNTHESIS AND CHARACTERIZATION OF A COMPLEMENTARY DNA PROBE FOR CHRYSANTHEMUM STUNT VIROID

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Received 26 May 1978

1. Introduction

Recent advances concerning the nature of viroid structure [1–3] and replication [4] have emphasized the need for a reliable probe for viroid sequences. The immediate potential use for such a probe is: (1) to look for sequence homology between different viroids and/or 'strains' or isolates of the same viroid; (2) to look for viroid sequences in the DNA of both healthy and infected plants; and (3) to study the site(s), rate and mode of viroid biosynthesis and in vivo processing.

Of the six well-known viroids [3], we have chosen chrysanthemum stunt viroid (CSV) as a model to study viroid replication. Like the other viroids, CSV is a single-stranded, highly base-paired, circular RNA molecule [1,3,5]. This unusual physical conformation has hindered the development of probes for viroid sequences.

We report here on a method for synthesizing complementary DNA (cDNA) probes to viroids, using CSV as our model. The probe has been characterized and shown to be specific for CSV sequences.

2. Materials and methods

CSV RNA was purified and characterized as described [5]. Nuclease-S_1 digestion of CSV RNA was performed as follows: 3–20 μg of CSV RNA in 25–40 μl of 0.03 M sodium acetate, 0.05 M NaCl, 0.001 M $ZnSO_4$, 5% (v/v) glycerol, pH 4.6, was incubated for 2 h at 25°C with nuclease S_1 at 1000 units/ml. The reaction was terminated and the RNA isolated by phenol:chloroform (1:1) extraction, three ether extractions and an ethanol precipitation. Polyadenylation of the RNA, preparation of nuclease S_1, preparation and isolation of cDNA, size estimation of the cDNA, hybridizations, thermal denaturation of hybrids and assay of hybrids formed by nuclease S_1, were performed as described in [6 and 7].

The RNAs of alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), and tobacco mosaic virus (TMV), were obtained as described in [8–10], respectively. Brome mosaic virus was kindly provided by Dr A. O. Jackson. *E. coli* B tRNA was obtained from Schwarz/Mann.

Nucleic acids (DNA and low mol. wt RNA) were extracted from chrysanthemums and gynuras *(Gynura aurantiaca)* as described [5]. Hybridizations using plant nucleic acid extracts were carried out to a Rot (moles of ribonucleotide/litre × time of hybridization in seconds) value high enough to detect sequence homology with the low mol. wt RNA, but not with the DNA.

3. Results

3.1. *Conditions for synthesis of cDNA probe*

The approach used to synthesize a cDNA probe to CSV was: (1) partial cleavage of CSV by nuclease-S_1; (2) enzymic addition of a poly(A) tract to the 3′-end of the S_1-treated CSV; and (3) synthesis of cDNA

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Table 1
Conditions for CSV cDNA synthesis

RNA template	Oligo(dT)primer	ng cDNA
S_1-treated CSV	–	0
	+	0.23
Polyadenylated, S_1-treated CSV	–	0.71
	+	7.02

S_1-treated CSV RNA was either used directly for cDNA synthesis, or was first polyadenylated; both as described in [7]. The RNA (0.8 μg) was divided into two fractions, to one of which was added 2 μg oligo(dT). cDNA yield is expressed in ng of cDNA synthesized/400 ng CSV RNA template

with reverse transcriptase, using polyadenylated, S_1-treated CSV as a template and oligo (dT) as a primer.

After treatment with nuclease-S_1, the extent of polyadenylation of CSV RNA increased such that 70% of the added ATP was incorporated into poly(A) tails, compared with 5% obtained without nuclease-S_1 treatment.

Table 1 shows that only the addition of a poly(A) tail and an oligo(dT) primer resulted in any appreciable cDNA synthesis. S_1-cleaved CSV was not capable of self-priming cDNA synthesis, and did not appear to contain any natural oligo(A) or oligo(U) sequences capable of binding to the oligo(dT) or poly(A) tails, respectively, which could then prime cDNA synthesis.

cDNA could also be made to polyadenylated CSV RNA which had not been treated previously with nuclease-S_1; however, the yield of cDNA was low (5% of that obtained with prior nuclease-S_1 treatment), and this cDNA proved to be unsuitable for hybridization purposes (results not shown).

3.2. *Characterization of the cDNA*

The cDNA was heterogeneous in size (fig.1) with most of the cDNA in the 20–100 000 daltons size range. Only a small proportion of the cDNA was full length (100–110 000).

The cDNA specifically hybridized only to CSV RNA (table 2), and not to any of the plant viral RNAs tested, or to *E. coli* tRNA. Therefore, not only was the cDNA probe specific for CSV RNA, but CSV appeared to have no sequence homology with the RNAs of either AMV, BMV, CMV or TMV.

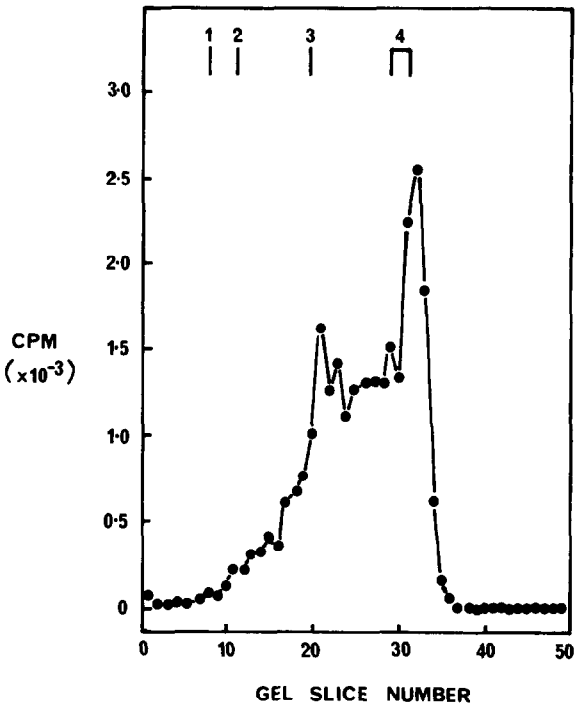

Fig.1. Size estimation of [^{32}P]cDNA to CSV by electrophoresis on 99% formamide: 5% polyacrylamide tube gels as described in [6]. The gel was cut up into 2 mm slices and counted by Cerenkov radiation. RNA markers were: (1) 16 S rRNA (5.5×10^5); (2) CMV RNA 4 (3.5×10^5); (3) CSV (1×10^5); and (4) *E. coli* tRNA ($2.2–2.9 \times 10^4$).

The specificity of the probe was further demonstrated by the ability of the cDNA to find complementary sequences only in nucleic acids (low mol. wt RNA) extracted from CSV-infected chrysanthemums or gynuras, but not from healthy chrysanthemums or gynuras or from citrus exocortis viroid (CEV)-infected gynuras (table 2).

The low maximum level of hybridization (50–64%) could be due to the short size of some of the cDNA (15–30 000 dalton range). cDNA of shorter size and/or low G:C content forms less stable hybrids; both factors lower the Tm of a hybrid [11,12]. This was supported by the thermal denaturation curve of the hybrid (fig.2), in which the curve was broader than the one obtained with the satellite RNA of CMV (SAT-CMV) and its cDNA, and the Tm was lower (80°C cf. 87°C obtained for SAT-CMV [7]). However, the shape of the curve was not as broad as the

Table 2
Specificity of CSV cDNA

Nucleic acid	Concentration (mg/ml)	% Hybridization
CSV	0.01	64.7
None	–	6.6
E. coli tRNA	1.25	6.8
AMV	1.25	6.8
BMV	1.25	8.2
CMV	1.25	9.3
TMV	1.25	12.3
Healthy chrysanthemum	2.0	12.3
CSV-infected chrysanthemum	2.0	48.9
Healthy gynura	2.0	10.6
CSV-infected gynura	2.0	61.2
CEV-infected gynura	2.0	12.8

Hybridization of [^{32}P]cDNA to CSV (0.8–1.6 ng/ml) with each of the RNAs was carried out to a Rot of 2.5 mol s l^{-1} (CSV), 320 (*E. coli* and viral RNAs), or 100 (plant nucleic acids) as described in [6]. Nuclease-S_1 assay of hybrids was as in [6], except that: (1) 0.3 M NaCl, (2) 10 units of nuclease S_1/assay, and (3) incubation at 37°C for 45 min were used

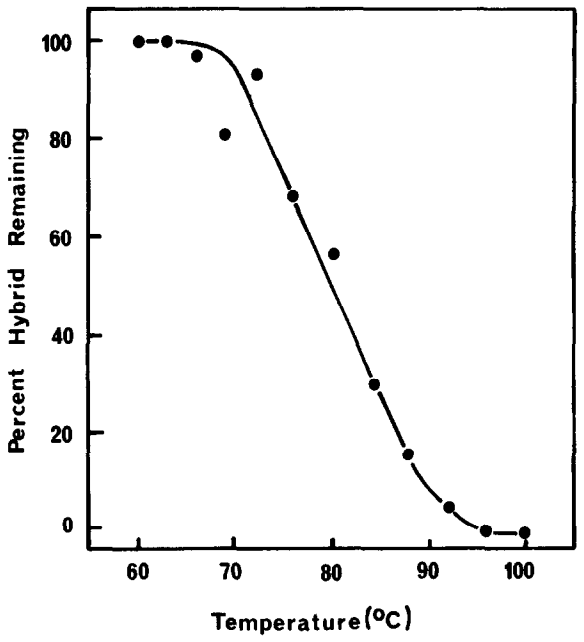

Fig.2. Thermal denaturation of [^{32}P]cDNA to CSV: CSV RNA hybrids in 0.01 M Tris–HCl, pH 7.0, 0.18 M NaCl, 0.001 M EDTA, 0.05% SDS, performed as described in [6].

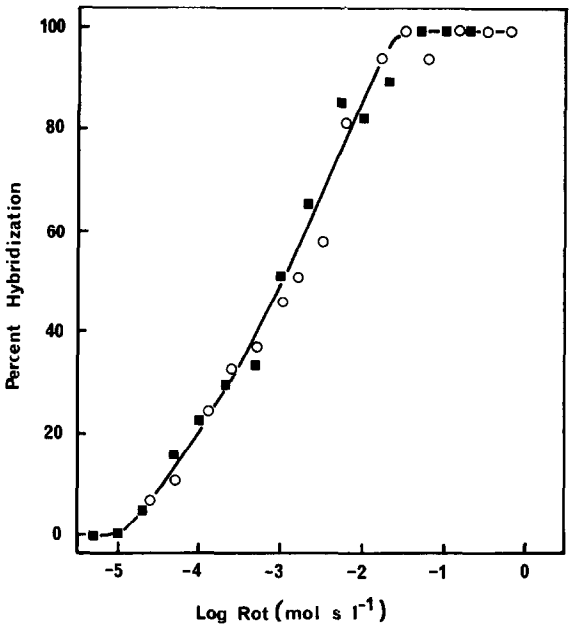

Fig.3. Kinetics of hybridization of [^{32}P]cDNA CSV with nuclease-S_1-treated CSV RNA under conditions of RNA excess. Hybridization and nuclease S_1 assay of hybrids were performed as described in [6]. Two different preparations of viroid were treated with nuclease-S_1 and the fragments were used in hybridizations with cDNA synthesized from the respective RNA preparation. The Rot curves have been normalized to facilitate comparison of the Rot½'s. The plateau values for ○ and ■ were 61% and 45%, respectively.

melting profiles observed with hybrids that were greatly mismatched [11,13], indicating little, if any, mismatching.

The kinetics of hybridization were examined by Rot analysis (fig.3). Since the rate of hybridization is proportional to the molecular complexity of the RNA [6,11], comparison of the Rot½ (the mid-point of the Rot curve) with that of a standard RNA of known complexity can reveal whether an RNA is a unique species or consists of multiple species with a similar mol. wt. The Rot½ for SAT-CMV (mol. wt 100–120 000) hybridized under the same conditions [7] was 10^{-3} mol s 1^{-1}, and SAT-CMV was shown to be a unique species. Therefore, CSV (Rot½ of 10^{-3}; mol. wt approx. 100 000 [5]) is a unique species of RNA and not a population of different molecules. This result was consistent with the fingerprint data on CSV [3].

4. Discussion

The cDNA probe hybridized only to CSV RNA and not to any of the other plant viral RNAs. Since the CMV RNA tested contained SAT-CMV, which was known to have several properties in common with viroids [14,15], this further demonstrated the absence of any detectable sequence homology between CSV and a satellite RNA.

Although the maximum level of hybridization of this cDNA probe (50–64%) and the level of nuclease-S_1 resistance (6–13%) were similar to those obtained for potato spindle tuber viroid (PSTV) cDNA synthesized by Hadidi et al. [16], the latter cDNA was not shown to be specific for PSTV sequences present only in the RNA of infected (but not healthy) plants; the cDNA hybridized to the RNA of satellite tobacco ringspot virus to the same extent as it did to PSTV; and no estimates as to the thermal stability of the hybrid or the molecular complexity of the RNA were made.

In contrast, the cDNA probe described here has been shown to be specific for CSV RNA: the probe was able to detect CSV sequences in nucleic acids extracted from two plant species infected with CSV (chrysanthemums and gynuras), but not from the respectively healthy plants. This provides further evidence for the ability of CSV to replicate in gynuras [5]. Furthermore, the probe did not appreciably hybridize to nucleic acids extracted from CEV-infected gynuras, indicating very little detectable sequence homology between the two viroids; a result confirmed by the 'fingerprints' of CSV and CEV [3]. The latter result also implies that inoculation of a host with one viroid does not lead to the stimulation of the host DNA to produce a second viroid; a result consistent with recent fingerprint data on PSTV and CEV [17]. This apparent lack of gross homology between viroids is of great importance, because it suggests that any small regions of homology between different viroids may be due to either a common binding site for a replicase, or related to the 'expression' of the viroid, i.e., its pathogenic effects.

Acknowledgements

The authors thank Dr R. I. B. Francki for the use of greenhouse facilities; Miss J. L. Rosey for technical assistance; and the Office of Program Resources and Logistics, Viral Cancer Program, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, MD, 20014, for providing avian myeloblastosis virus reverse transcriptase. This work was supported by the Australian Research Grants Committee.

References

[1] Sänger, H. L., Klotz, G., Riesner, D., Gross, H. J. and Kleinschmidt, A. K. (1976) Proc. Natl. Acad. Sci. USA 73, 3852–3856.
[2] Henco, K., Riesner, D. and Sänger, H. L. (1977) Nucleic Acids Res. 4, 177–194.
[3] Gross, H. J., Domdey, H. and Sänger, H. L. (1977) Nucleic Acids Res. 4, 2021–2028.
[4] Mühlbach, H.-P. and Sänger, H. L. (1977) J. Gen. Virol. 35, 377–386.
[5] Palukaitis, P. and Symons, R. H. Manuscript in preparation.
[6] Gould, A. R. and Symons, R. H. (1977) Nucleic Acids Res. 4, 3787-3802.
[7] Gould, A. R., Palukaitis, P., Symons, R. H. and Mossop, D. W. (1978) Virology, 84, 443–455.
[8] Van Vloten-Doting, L. and Jaspars, E. M. J. (1972) Virology 48, 699–708.
[9] Peden, K. W. C. and Symons, R. H. (1973) Virology 53, 487–492.

[10] McLean, G. D. and Francki, R. I. B. (1967) Virology 31, 585–591.
[11] Gillespie, D., Gillespie, S. and Wong-Staal, F. (1975) in: Methods in Cancer Research (Busch, H. ed.) 11, pp. 205–245, Academic Press, NY.
[12] Gould, A. R., personal communication.
[13] Gonda, T. J. and Symons, R. H. (1978) Virology, in press.
[14] Mossop, D. W. and Francki, R. I. B. (1978) Virology, in press.
[15] Mossop, D. W., personal communication.
[16] Hadidi, A., Diener, T. O. and Modak, M. J. (1977) FEBS Lett. 75, 123–127.
[17] Dickson, E., Diener, T. O. and Robertson, H. D. (1978) Proc. Natl. Acad. Sci. USA 75, 951–954.