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RNA sequences complementary to citrus exocortis viroid in nucleic acid preparations from infected *Gynura aurantiaca*

(molecular hybridization/RNA·RNA hybrid)

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ABSTRACT **Molecular hybridization with ^{125}I-labeled citrus exocortis viroid RNA has been used to survey nucleic acid preparations from *Gynura aurantiaca* for viroid complementary molecules. A differential hybridization effect was detected between nucleic acid extracts from healthy and infected tissue in which significant RNase-resistant ^{125}I-labeled citrus exocortis viroid resulted in hybridization studies with the infected tissue extracts. Subsequent characterization indicated that RNA from infected tissue was involved in the formation of a duplex molecule with citrus exocortis viroid RNA and had properties of an RNA·RNA hybrid. Subcellular fractionation of infected tissue indicates that the complementary RNA is present in nuclear and soluble RNA fractions. This RNA may represent an intermediate molecule in the replication of the viroid or a pathogenic expression and may have a regulatory role in the host cell.**

The exocortis disease has been shown to be induced by a low molecular weight (10^5) RNA viroid, citrus exocortis viroid (CEV), by Semancik and Weathers (1). All attempts to translate the RNA molecule *in vitro* as well as to detect a viroid-specified protein have been unsuccessful (2, 3). The limited coding capacity of the viroid suggests that its replication is highly dependent on host polymerase systems. An intermediate necessary to act as a template for the subsequent production of infectious RNA molecules would theoretically be RNA or DNA that may or may not exist in the host cell prior to infection. Previous attempts to detect a complementary form have indicated a high concentration of complementary sequences in DNA-rich preparations from the nuclear fractions of CEV-infected tissue (4).

Characterization of a hybrid formed between RNA preparations extracted from *Gynura aurantiaca* and ^{125}I-labeled CEV indicates that there is a viroid complementary nucleic acid which is RNA. This RNA species, which is present in the nuclear and high-speed supernatant extracts, may be an intermediate form of CEV. Its affinity for the nuclear fraction supports the suggestion that it may fulfill a regulatory role in the host cell, and may be responsible for viroid pathogenesis (5, 6).

MATERIALS AND METHODS

Preparation of CEV. Biologically active CEV RNA was isolated from a mixture of nucleic acids obtained by homogenizing infected *Gynura aurantiaca* DC in phenol, as reported by Semancik *et al.* (7). Included in this purification procedure was the isolation of the CEV RNA from polyacrylamide gels after electrophoresis of a highly purified preparation. The isolated infectious RNA was subsequently analyzed by electrophoresis in 5% polyacrylamide gels followed by staining, by the method of Morris and Wright (8). It was found to migrate as a single homogeneous band without any detectable contamination.

Preparation of Nucleic Acids. Nucleic acids were extracted from buffer- or CEV-inoculated plants 1–3 months after inoculation, as described by Semancik and Weathers (1). The phenol-extracted nucleic acid preparations were further purified by removing contaminating polysaccharides by the methoxyethanol procedure of Bellamy and Ralph (9) and by degrading contaminating proteins with the addition of proteinase K (Beckman) at a final concentration of 10 μg/ml followed by another phenol extraction. The purified nucleic acids were fractionated with 2 M LiCl to separate single-stranded nucleic acids lacking extensive self-complementarity from those with self-complementarity and from the double-stranded nucleic acids (10). Three volumes of 95% ethanol were added to the supernatant resulting from the salt fractionation procedure and the windable, precipitated nucleic acids were spooled out on a glass rod. Both the LiCl-precipitated nucleic acids and the DNA-rich windings were resuspended in and dialyzed against TKM buffer (10 mM Tris/10 mM KCl/0.1 mM $MgCl_2$ at pH 7.4), then stored at a concentration of 2 or 8 mg/ml at −20°.

Subcellular Fractionation. CEV-infected *Gynura* tissue was homogenized with a polytron homogenizer and fractionated by differential centrifugation (11). Subcellular fractions were extracted with phenol. The nucleic acids were then precipitated with ethanol, resuspended in TKM buffer, and partitioned in 2 M LiCl. After another ethanol precipitation, the fractions were resuspended and dialyzed against TKM buffer.

Iodination of RNA. RNA species were iodinated by E. Dickson (Rockefeller University) by the method of Prensky *et al.* (12). Four different preparations of CEV RNA, with specific activities of approximately 10^4 cpm/ng, were used in these studies. Bacteriophage f2, bean 18S, rabbit 9S, and tomato 5S RNAs were kindly provided by and also iodinated by E. Dickson with similar specific activities. Self-hybridization of all these RNAs was negligible under the conditions used in the hybridization experiments.

Hybridization Techniques. ^{125}I-labeled CEV RNA, at a final concentration of approximately 0.02 μg/ml, was incubated with nucleic acid preparations in 4× SSC and 50% formamide at 42°. (SSC 0.15 M NaCl/0.015 sodium citrate at pH 7.) These conditions minimize the breakdown of the iodinated RNA probe and have been shown to be optimal for the formation of an iodinated RNA·RNA hybrid (13). Prior to hybridization the nucleic acids were heated to 85° for 10 min in the formamide solution. At the termination of the experiment, two aliquots

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Abbreviations: CEV, citrus exocortis viroid; C_rt, initial concentration of RNA (mol of nucleotide per liter) × time (sec); SSC, 0.15 M sodium chloride/0.015 M sodium citrate at pH 7; × SSC is the concentration of the buffer used times that of SSC; ss RNA, single-stranded RNA; TKM buffer, 10 mM Tris/10 mM KCl/0.1 mM $MgCl_2$ at pH 7.4.

Table 1. Distribution of RNase-resistant ^{125}I-labeled CEV RNA after hybridization to subcellular nucleic acid fractions

Fraction	cpm using 2 M LiCl	
	Precipitate	Supernatant
Pellet: 750 × *g*, 10 min	1043	710
Pellet: 40,000 × *g*, 20 min	278	260
Pellet: 100,000 × *g*, 1 hr	111	108
Supernatant: 100,000 × *g*, 1 hr	1582	300

Hybridization conditions and nucleic acid fractionation techniques are as described in *Materials and Methods*. Nucleic acid concentrations were estimated from an extinction coefficient of 25 A_{260} units/ml·mg^{-1} as 1 mg/ml. With these values hybridization procedures were done with initial concentration of nucleic acids (mol of nucleotide per liter) × time (sec) equal to 100 (C_rt = 100). Each 50-μl aliquot of reaction mixture contained 10^4 cpm of ^{125}I-labeled CEV RNA. Background RNase-resistant cpm, as determined by a control without added RNA, was subtracted from each sample.

were removed; one was diluted in 30 volumes of sterile 2× SSC and the other in 30 volumes of 2× SSC containing RNase A (Worthington; 200 units/ml) and RNase T_1 (Worthington; 40 units/ml). Both aliquots were incubated at 30° for 45 min, after which bovine serum albumin was added to a final concentration of 50 μg/ml and the mixtures were made 10% (wt/vol) in trichloroacetic acid. After 30 min on ice, acid-precipitable material was collected on Whatman GF/C filters and the radioactivity was measured in Omnifluor/toluene scintillation solution in a Beckman LS-3133 scintillation counter. In the experiments a control was included in which iodinated CEV RNA was incubated under conditions of hybridization but without added RNA. The RNase-resistant ^{125}I-labeled CEV RNA in such cases ranged from 100 to 200 cpm, and in experiments indicated, this value was subtracted from the other hybridizations performed at that time. The products of hybridization experiments were recovered by precipitation after addition of three volumes of cold 95% ethanol to the reaction mixture. After 1 hr at −20°, the precipitate was collected by centrifugation (10,000 × *g*, 15 min), resuspended, and dialyzed against the desired buffer for subsequent use to determine the t_m (temperature at which 50% of the hybrid denatures) and RNase H (Miles Laboratories) sensitivity.

The data presented here are those resulting from a typical experiment, with all experiments being performed at least twice.

RESULTS

Hybridization of ^{125}I-Labeled CEV RNA to Subcellular Nucleic Acid Preparations. In a previous study, total nucleic acids extracted from subcellular fractions demonstrated that CEV RNA hybridized predominantly with nuclei-rich preparations (11). In that study, the type of nucleic acids to which CEV RNA hybridized was not determined. The DNA-rich nucleic acids contain a high amount of 4S, 5S, and CEV RNA, as well as DNA (14). In an attempt to determine the type of nucleic acids to which the CEV RNA hybridized, as well as the distribution of the molecule, hybridization with LiCl-fractionated nucleic acid species from subcellular components was investigated. Tissue derived from CEV-infected *Gynura* was subjected to subcellular fractionation; then the nucleic acids were extracted and fractionated by LiCl precipitation as described. The LiCl precipitates consist of essentially purified single-stranded RNA (ss RNA), since virtually no DNA can be detected by the diphenylamine test, whereas the nucleic acids soluble in 2 M LiCl are more heterogeneous and consist largely of partially duplex RNA and double-stranded nucleic acids. The ability of each of these fractions to hybridize with CEV ^{125}I-labeled RNA is presented in Table 1. The largest amount of hybridization occurred with the LiCl-precipitated nucleic acids of the 100,000 × *g* supernatant material, suggesting that the ^{125}I-labeled CEV RNA is hybridizing to a ss RNA. Significant amounts of hybrid were formed with both the LiCl precipitate and supernatant material of the 750 × *g* nuclei-rich fraction, indicating a subcellular distribution of the nucleic acid complementary to viroid RNA, possibly with different properties. The LiCl-precipitable complementary nucleic acid is distinct from the viroid RNA, which remains predominantly in the LiCl supernatant material (11).

Table 2. Hybridization of various ^{125}I-labeled RNAs with 2 M LiCl-precipitated nucleic acids from healthy and CEV-infected *Gynura*

^{125}I-Labeled RNA source	RNase-resistant ^{125}I-labeled RNA cpm	
	Healthy	CEV-infected
CEV	205	785
Bacteriophage f2	165	152
Bean 18S rRNA	142	156
Rabbit 9S mRNA	150	153
Tomato 5S rRNA	150	161

Conditions of hybridization are as described in *Materials and Methods*. In all experiments a C_rt value of 100 was used. Each 50-μl aliquot of reaction mixture contained 6 × 10^3 cpm of ^{125}I-labeled RNA.

Characterization of Hybridization Reaction. To establish the specificity of the CEV RNA probe, ^{125}I-labeled RNAs of varying size and complexity were hybridized with nucleic acids from healthy and CEV-infected *Gynura*. The results (Table 2) demonstrate that the differential effect prominent with the ^{125}I-labeled CEV RNA probe was not observed with the other ^{125}I-labeled RNAs. These data support the likelihood that the interaction between the CEV RNA probe and the nucleic acids from the infected host tissue is specific.

Factors affecting the efficiency of the CEV RNA hybridization with *Gynura* LiCl-precipitated nucleic acids were at optimal levels in the standard protocol (Fig. 1). The experiments showed that the efficiency of the hybridization reaction was dependent on formamide and SSC concentration, as well as time.

The kinetics of hybridization were observed with a fixed amount (0.02 μg/ml) of ^{125}I-labeled CEV RNA and various amounts of LiCl-precipitable nucleic acids from whole *Gynura* leaf tissue (Fig. 2). Saturation was reached at a C_rt of approximately 600, with a recovery of about 30% of the input ^{125}I-labeled CEV RNA.

Properties of the Hybrid. Since precipitation with 2 M LiCl yields a nucleic acid preparation consisting essentially of ss RNA and since the LiCl-soluble nucleic acids contain RNA as well as DNA, the possibility that the CEV RNA might be hybridizing with complementary RNA became more probable. Treatment of the LiCl precipitate with RNase A before hybridization abolished its ability to hybridize with CEV RNA. However, similar treatment with DNase I had no effect on the amount of hybrid formed, demonstrating that RNA is primarily responsible for hybridization observed with LiCl-precipitated nucleic acids (Table 3). In addition, the incubation of the LiCl-precipitated nucleic acids in 0.3 M NaOH for 24 hr followed by exhaustive dialysis against TKM buffer also abolished the ability of the nucleic acid preparation to hybridize with the

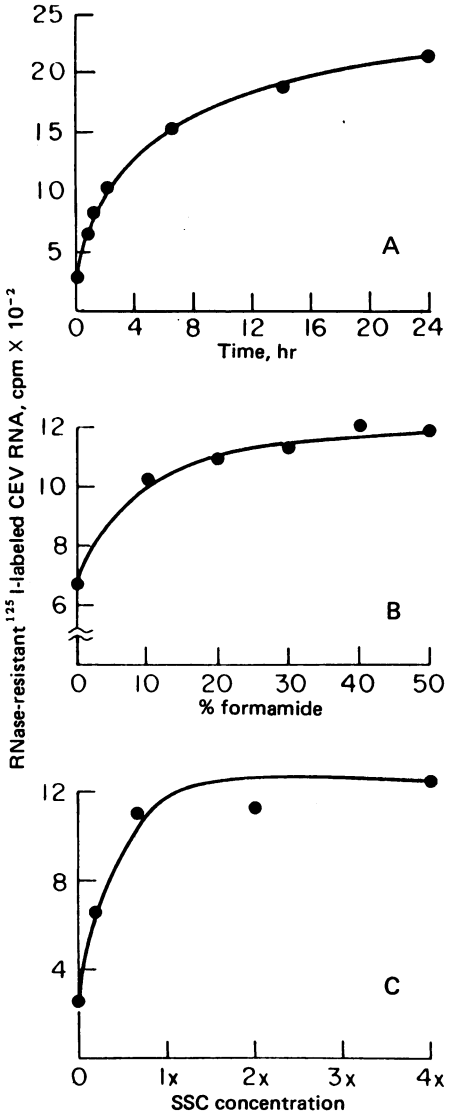

FIG. 1. (*A*) Hybridization with 2 M LiCl-precipitated nucleic acids from CEV-infected *Gynura* at a concentration of 2.4 mg/ml. (*B*) Dependence of hybridization on formamide concentration, with 2 M LiCl-precipitated nucleic acids at a C_rt of 100. Hybridization conditions were as in *Materials and Methods* except that varying concentrations of formamide were used. (*C*) Dependence on SSC for the formation of the hybrid. Conditions are as described in *B*, but the SSC concentration was varied and the formamide concentration was maintained at 50%. Each 50-μl aliquot of reaction mixture contained 6×10^3 cpm of ^{125}I-labeled CEV RNA.

iodinated CEV RNA, thereby further supporting the existence of a RNA viroid complement.

Isolation of the hybrid by ethanol precipitation and treatment with RNase H failed to degrade the molecule, while under identical conditions, a synthetic poly(dT)·[^{3}H]poly(U) hybrid (Miles Laboratories) was rapidly degraded, reducing the possibility that an RNA·DNA hybrid is formed.

The denaturation profile of the hybrid formed between ^{125}I-labeled CEV RNA and the LiCl-precipitated nucleic acids is presented in Fig. 3. The hybrid formed is characteristic of double-stranded RNA with a t_m of 90° in 0.01× SSC and a t_m of 95° in 0.1× SSC. These high t_m values, expected for a duplex RNA molecule rich in G − C pairs, are predicted by the high G + C content of CEV (7).

Although CEV displays a degree of self-homology (15), this

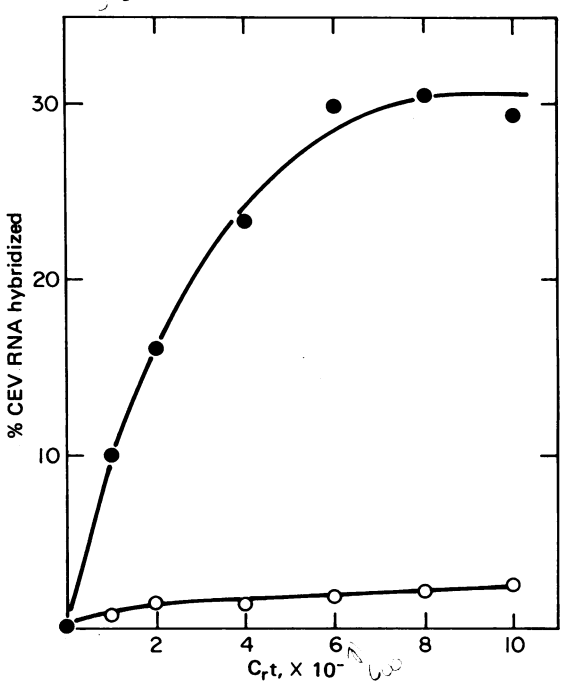

FIG. 2. Kinetics of hybridization between ^{125}I-labeled CEV RNA and the 2 M LiCl-precipitated nucleic acids from healthy (○) and CEV-infected (●) *Gynura*. Conditions of hybridization were as described in *Materials and Methods*, with only the RNA concentration varied. Background RNase-resistant cpm, as determined by a control consisting of ^{125}I-labeled CEV RNA without added RNA, was subtracted from each sample.

does not seem to affect the hybridization studies since high amounts of purified unlabeled CEV can be added to healthy LiCl-extracted nucleic acids before hybridization and there is no increase in hybridizable ^{125}I-labeled CEV RNA above background. Furthermore, the addition of high amounts of purified unlabeled CEV to CEV-infected, LiCl-extracted nucleic acids compete with ^{125}I-labeled CEV RNA in the formation of the hybrid.

DISCUSSION

These hybridization studies were performed under conditions chosen to minimize the background of nonspecific hybridization by avoiding the degradation of the nucleic acid preparations and the ^{125}I-labeled probe. Higher hybridization temperatures in the absence of formamide have been shown to promote the breakdown of the ^{125}I-labeled RNA to pieces smaller than 4 S (13). The data presented here demonstrate that the conditions used appear optimal for hybrid formation with CEV RNA and prevent the degradation of the iodinated probe.

It has previously been demonstrated that DNA-rich preparations from CEV-infected *Gynura* hybridize to ^{125}I-labeled CEV more extensively than similar extracts from healthy tissue (4). This effect has been confirmed under a number of different conditions as well as on an alternate host (unpublished data). The hybridization studies with subcellular fractions resulted in significant amounts of hybrid formed with the LiCl-precipitable and the LiCl-soluble nucleic acids of the nuclei-rich fraction, and also in the LiCl-precipitable fraction of the 100,000 × *g* supernatant material from the cellular homogenate. The hybridization of ^{125}I-labeled CEV RNA to LiCl-precipitated RNA from the nuclear fraction gives evidence that there is a RNA viroid complement in the nucleus. The hybrid formation with the 100,000 × *g* supernatant material also

Table 3. Hybridization of ^{125}I-labeled CEV RNA with 2 M LiCl-precipitated nucleic acids, treated with RNase or DNase, from CEV-infected *Gynura*

LiCl precipitate hybridized	RNase-resistant ^{125}I-labeled CEV RNA, cpm
Healthy; no treatment	138
Infected; no treatment	1330
Infected; DNase-treated	1243
Infected; RNase-treated	254

Prior to hybridization, equal aliquots were incubated 2 hr at 30° in 0.2 ml of TKM buffer containing, as indicated, DNase I (Worthington; 10 units) or RNase A (40 units). Proteinase K (Beckman) was then added to a final concentration of 0.1 mg/ml to degrade the RNase or DNase. After 1 hr at 20° the nucleic acids were extracted by addition of two volumes of phenol and centrifugation in a Beckman Microfuge B. Equal volumes of the resulting aqueous (upper) phase from each were used in the hybridization studies. Each 50-μl aliquot of reaction mixture contained 5×10^3 cpm of ^{125}I-labeled CEV RNA.

suggests that cellular CEV is not involved in the hybridization with the labeled CEV due to CEV RNA properties of self-homology, since CEV infectivity resides primarily in the nuclei-rich preparation and is absent in the 100,000 × *g* supernatant material (11).

The enzymatic treatments showing the complementary molecular to be sensitive to RNase but not DNase, along with a melting profile similar to ϕ6 double-stranded RNA (7) verify

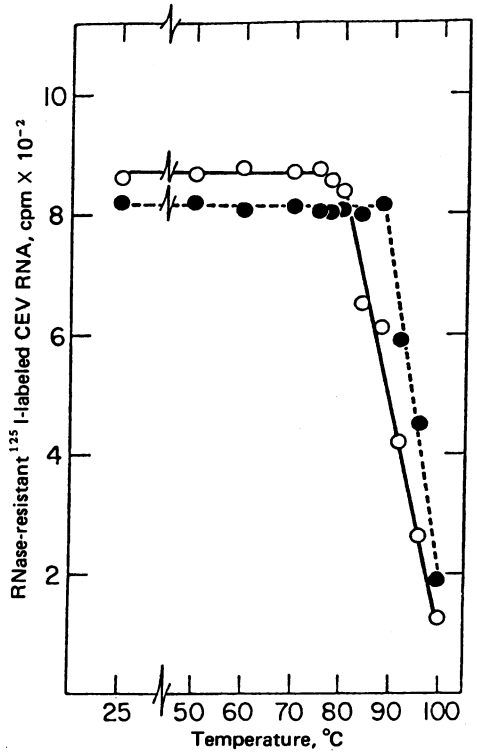

FIG. 3. Thermal stability of the hybrid formed between ^{125}I-labeled CEV RNA and the 2 M LiCl-precipitated nucleic acid fraction from CEV-infected *Gynura*. Hybridizations were performed at a C_rt of 500. The nucleic acids were precipitated by addition of three volumes of 95% ethanol then resuspended in either 0.01 × SSC (○) or 0.1 × SSC (●) and dialyzed against the respective buffer overnight. Aliquots were incubated at temperatures ranging from 25° to 100° for 10 min each. Forty volumes of 2 × SSC buffer containing RNase A (200 units/ml) and RNase T_1 (40 units/ml) were added and the mixture was incubated at 30° for 1 hr. The RNase-resistant trichloroacetic acid-precipitable cpm were collected and measured.

that the product is an RNA·RNA hybrid. Since LiCl treatment is capable of precipitating the viroid complementary RNA molecule, it may have a higher molecular weight than CEV or lack significant secondary structure characteristic of CEV. This complementary RNA could consist of single or multiple copies of the sequence complementary to CEV or it may be a larger RNA molecule of which the CEV complementary sequence constitutes only a small part. The hybrid formed from both the LiCl-precipitable and the LiCl-soluble nucleic acids in the nuclei-rich fraction may result from more than one species of complementary RNA, such as single and tandemly repeated copies of the CEV complementary, or fragmented pieces initially from larger heterogeneous molecules that still remain. It is also possible that the RNA remains entrapped or associated with the DNA such that it cannot be precipitated by LiCl.

The hybridization studies of the potato spindle tuber viroid of Hadidi *et al.* (16) did not show a differential hybridization effect between healthy and viroid-infected NaOH-treated, DNA-rich nucleic acids from tomatoes. In view of the data presented here, indicating that sequences complementary to CEV in the infected tissue are ss RNA, it would be expected that DNA purification techniques involving NaOH hydrolysis would destroy the complementary RNA and therefore the differential effect. That the study with potato spindle tuber viroid did not reveal an RNA complementary to the viroid might be due to the fact that the conditions used for isolation of RNA were not optimal for ss RNA or that the potato spindle tuber viroid-tomato system differs somewhat from the CEV-*Gynura* system.

The role of the complementary RNA might be that of replication of the CEV and/or that of a regulatory role involved in the pathogenic expression. The replicative role might indicate that the viroid infection process involves a host-mediated replication mechanism by which CEV RNA complement and subsequent CEV RNA are synthesized. That a regulatory role may exist in the CEV infection process, causing pathogenesis, has been proposed (5, 6). This role could involve the complementary RNA as well as the CEV itself. The fact that the viroid complementary molecule is found in the LiCl-precipitated RNA of the nuclei-rich fraction along with the properties of the exocortis viroid pathogenesis (17), suggest that the complementary form of CEV RNA may be analogous to an activator RNA molecule involved in gene regulation, as proposed by Britten and Davidson (18), or a regulatory informational carrier involved in communication between the cell surface and the DNA, as proposed by Dickson and Robertson (19). However, verification of this possibility must await further characterization of the complementary RNAs in the nuclear fraction and the high-speed supernatant material and the elucidation of the function of the complementary RNA.

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1. Semancik, J. S. & Weathers, L. G. (1972) *Virology* **47**, 456–466.
2. Hall, T. C., Wepprich, R. K., Davies, J. W., Weathers, L. G. & Semancik, J. S. (1974) *Virology* **61**, 486–492.
3. Zaitlin, M. & Hariharasubramanian, V. (1972) *Virology* **47**, 296–305.
4. Semancik, J. S. & Geelen, J. L. M. C. (1975) *Nature* **256**, 753–756.
5. Semancik, J. S. & Weathers, L. G. (1972) *Virology* **49**, 622–625.

6. Semancik, J. S. & Weathers, L. G. (1972) *Nature New Biol.* **237,** 242–244.
7. Semancik, J. S., Morris, T. J., Weathers, L. G., Rodorf, B. F. & Kearns, D. R. (1975) *Virology* **63,** 160–167.
8. Morris, T. J. & Wright, N. S. (1975) *Am. Potato J.* **52,** 57–63.
9. Bellamy, A. R. & Ralph, R. K. (1968) in *Methods in Enzymology,* eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 12B, pp. 156–160.
10. Baltimore, D. (1966) *J. Mol. Biol.* **18,** 421–428.
11. Semancik, J. S., Tsuruda, D., Zaner, L., Geelen, J. L. M. C. & Weathers, L. G. (1976) *Virology* **69,** 669–676.
12. Prensky, W., Steffensen, D. M. & Hughes, W. L. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 1860–1864.
13. Farace, M.-G., Aellen, M.-F., Briand, P.-A., Faust, C. H., Vassalli, P. & Mach, B. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 727–731.
14. Semancik, J. S., Conejero, V. & Gerhart, H. (1977) *Virology* **80,** 218–221.
15. Semancik, J. S., Morris, T. J. & Weathers, L. G. (1973) *Virology* **53,** 448–456.
16. Hadidi, A., Jones, D. M., Gillespie, D. H., Wong-Staal, F. & Diener, T. O. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2453–2457.
17. Conejero, V. & Semancik, J. S. (1977) *Virology* **77,** 221–232.
18. Britten, R. J. & Davidson, E. H. (1969) *Science* **165,** 349–357.
19. Dickson, E. & Robertson, H. D. (1976) *Cancer Res.* **36,** 3387–3393.