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20. We thank R. Evart for help with the hepatectomies, S. Neil for photographic assistance, and F. Williams and A. Gaddis for secretarial help.

21 January 1987; accepted 7 April 1987

In Vivo Uncoating and Efficient Expression of Foreign mRNAs Packaged in TMV-Like Particles

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The ribonucleocapsids of many plant viruses are extremely stable. The protein coat protects the RNA genome against degradation during the accumulation and spread of progeny virions. Chimeric single-stranded RNA molecules were transcribed in vitro from recombinant plasmids and later encapsidated, in vitro, into ribonucleoprotein particles (pseudoviruses) 60 nanometers long that resembled tobacco mosaic virus. Transcripts encoding an assayable enzyme, chloramphenicol acetyltransferase (CAT), were packaged into pseudovirus particles to assess the utility of this single-stranded RNA delivery system in a wide range of cell types. In all cases, packaged CAT messenger RNA was uncoated and transiently expressed. Significantly higher levels of CAT activity were detected with packaged than with naked CAT messenger RNA after inoculation of plant protoplasts in the presence of polyethylene glycol or abrasive inoculation of intact leaf surfaces. Structural events that lead to the uncoating and expression of CAT messenger RNA showed no cell specificity. This observation may support the view that the comparatively restricted host range of a true plant virus results from events that occur later during the infection cycle.

NUCLEOPROTEIN STRUCTURES OF many viruses, particularly plant viruses, are extremely stable under adverse environmental conditions. The capsid protein protects the genome (DNA or, more especially, RNA) from ubiquitous nucleases during the intracellular accumulation, release, and spread of progeny virions. A number of animal pseudovirus vector systems have been developed (*1–3*) to exploit, in part, this protective function; more importantly, these vector systems increase the efficiency of gene transfer by utilizing the receptor-mediated cell-attachment and uptake properties of animal viruses. Although there is no evidence for such a specific uptake mechanism among plant viruses, we were interested in developing and testing, in vivo, an RNA delivery-expression system based on a well-characterized plant RNA virus, tobacco mosaic virus (TMV) (*4*). The ribonucleocapsids of many plant or animal viruses appear to remain virtually intact as they enter the cytoplasm of the newly infected host cell (*5*). Although this may ensure some continued protection for the messenger-sense viral RNA, it presents a paradox: how is the genome released from such a functionally stable structure under the comparatively mild physico-chemical conditions of the cytoplasm? Some recent evidence suggests that ribosomes may be involved in a process of cotranslational disassembly (*6–8*). To extend our studies on this process in vivo, and to develop a potentially useful protective system for the encapsidation, delivery, and transient expression of single-stranded RNA (ssRNA) molecules in a wide range of cells, we undertook the studies described in this report.

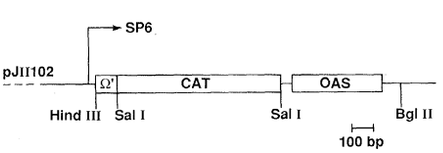

Fig. 1. Portion of the in vitro transcription plasmid pJII102 (*10*) showing the orientation and transcription start point of the bacteriophage SP6 promoter (arrowhead above), the CAT-coding region (0.78 kb), the OAS (0.45 kb) from TMV (U1 strain), and relevant restriction enzyme sites used for insertion into (Hind III and Sal I), or linearization of (Bgl II), the template.

Preliminary work on an SP6-transcript packaging vector has been described elsewhere (*9*). Recently, we reported (*10*) that expression of unencapsidated chloramphenicol acetyltransferase (CAT) messenger RNA (mRNA) is enhanced, both in vitro and in vivo, by the presence of either a 5′-cap structure or a derivative of the 5′-untranslated leader sequence of TMV RNA (Ω′), or both.

Plasmids pJII102 (Fig. 1) and pJII2 (*10*) contained the CAT-coding region from Tn9 (*11*), either with or without the modified 5′-leader sequence (Ω′) from TMV RNA (U1 strain). In vitro transcription of linearized forms of these plasmids resulted in RNA molecules with the TMV (U1) origin-of-assembly sequence (OAS) at the 3′ end. Uncapped or 5′-capped forms of each of these transcripts could be assembled into TMV-like pseudovirus particles (Fig. 2A) 60 nm in length (Fig. 2B), as predicted from the size of the in vitro transcript (1.2 kb) and by comparison with 300-nm-long TMV particles which contain a 6.4-kb genome. By means of ^{32}P-labeled transcripts, it was pos-

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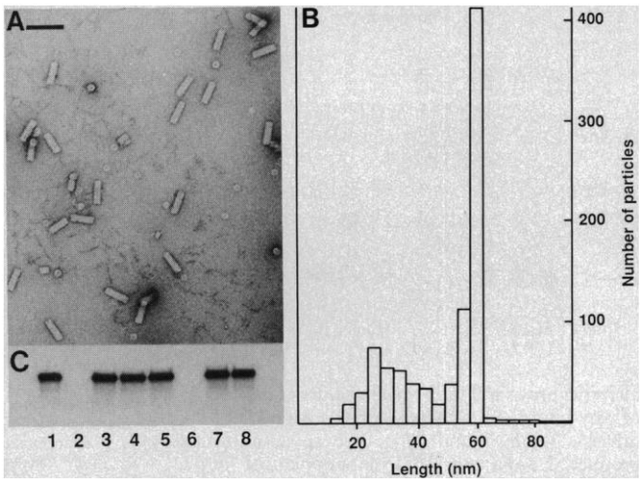

Fig. 2. Characterization of pseudovirus particles containing CAT mRNA. (**A**) Electron micrograph of ribonucleoprotein particles recovered by centrifugation after an in vitro encapsidation reaction containing SP6 transcripts of Bgl II–linearized pJII102. Scale bar, 100 nm. (**B**) Histogram showing the length distribution of particles on micrographs as in (A). The modal length of CAT pseudoviruses, predicted on the basis of the size of the RNA transcript (1.2 kb), would be 60 nm. (**C**) Autoradiograph of a 1.5% (w/v) agarose gel after electrophoresis of ^{32}P-labeled CAT-OAS transcripts before (lanes 1 and 5) or after incubation with micrococcal nuclease (100 unit/ml) for 30 minutes (lanes 2 and 6) at 20°C. Radiolabeled transcripts were incubated with TMV protein (lanes 3 and 7) and later subjected to micrococcal nuclease (lanes 4 and 8). Transcripts shown in lanes 5 through 8 contained anti-sense CAT mRNA attached to the conventionally oriented 3′-OAS.

sible to demonstrate that encapsidation provided complete protection against ribonuclease (RNase) treatment (Fig. 2C). Inversion of the CAT-coding sequence alone had no effect on either the efficiency of encapsidation or the extent of transcript protection (Fig. 2C, lanes 5 through 8), confirming the sequence-independent feature of this RNA-packaging system.

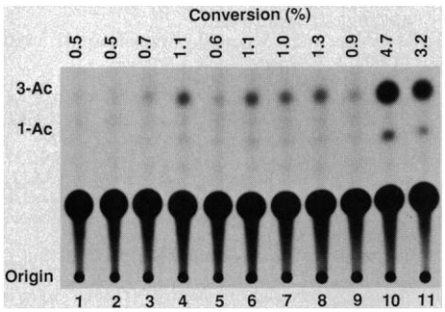

Fig. 3. Thin-layer chromatographic (TLC) assay for CAT activity in extracts from PEG-inoculated tobacco mesophyll protoplasts (*12*). Protoplasts were mock-inoculated with PEG alone (lane 1) or received equivalent amounts (on an RNA weight basis) of TMV (lane 2), naked CAT mRNA (lane 3), CAT pseudovirus particles (lane 4), 5′-capped–CAT mRNA (lane 5), 5′-capped–CAT pseudovirus (lane 6), Ω′-CAT mRNA (lane 7), Ω′-CAT pseudovirus (lane 8), 5′-capped–Ω′-CAT mRNA (lane 9), or 5′-capped–Ω′-CAT pseudovirus particles (lane 10) and were incubated at 25°C for 20 hours. The protoplasts were removed from isotonic culture medium (*12*) by centrifugation, then resuspended and sonicated (10 seconds) in an equal volume of 0.25*M* tris-HCl, *p*H 7.4, containing 10 m*M* dithiothreitol (DTT) and 2 m*M* leupeptin. Cellular debris was removed by centrifugation at 10,000*g* for 10 minutes at 4°C and 100-μl samples of each supernatant were assayed for CAT activity. CAT assays were performed as described (*10, 13*). As a reference, 0.025 unit of purified CAT was added to a sample of the mock-inoculated extract (lane 11). The TLC plate was autoradiographed for 30 hours at room temperature. Positions of the 1- or 3-monoacetylated derivatives of [^{14}C]chloramphenicol are shown on the left.

Pseudovirus particles containing CAT mRNAs with different leader sequences, or the unencapsidated CAT mRNAs themselves, were used to "pseudo-infect" tobacco mesophyll protoplasts by the polyethylene glycol (PEG)–inoculation technique (*12*). Excess inoculum was removed and the protoplasts were incubated for 20 hours; protoplast extracts were then assayed for CAT activity (*13*). As demonstrated in other experiments (*10*), addition of both a 5′-cap structure and the TMV-leader sequence (Ω′) enhanced the expression of CAT significantly more (in this case, about fourfold more) than either feature alone (Fig. 3). However, in PEG-inoculated protoplasts, this effect was apparent only with the packaged forms of the CAT mRNAs and may be attributed to the variable efficiency of recruitment of ribosomes onto fully uncoated CAT mRNAs, released independently of translation in vivo. Alternatively, these 5′ sequences may have some direct, translationally linked, effect on the initiation of particle disassembly inside cells. It is unlikely that the unencapsidated CAT mRNAs survived the inoculation procedure (Fig. 3, lanes 3, 5, 7, and 9).

Tobacco cells are natural hosts for TMV. Therefore pseudo-TMV particles might be expected to undergo analogous, although undefined, "early events" in this system. Pea (*Pisum sativum L*), on the other hand, is classified as one of the poorest "subliminal" hosts for TMV (*14*). TMV particles inoculated onto the epidermal cells of tobacco (*7*) or pea (*8*) become partially uncoated and appear to form intermediate translation complexes called "striposomes" (*6*). Studies with peas were facilitated by use of a mutant called *Argenteum* (*15*), which has an easily peeled epidermis. We chose to examine CAT expression in epidermal cells of *Argenteum* pea by inoculating pseudovirus particles, or equivalent amounts of unencapsidated CAT mRNA constructs, directly onto the leaf surface with silicon carbide (Carborundum, 180 grit) as an abrasive (Fig. 4). In each case, RNA encapsidation increased the level of CAT expression. This was particularly evident with the 5′-capped–Ω′-CAT construct. When the absolute amounts (counts per minute) of 3-acetyl-[^{14}C]chloramphenicol product were compared, rather than the activity values expressed as percent conversion, the encapsidated form of this construct resulted in 65 times more CAT activity than the equivalent amount of naked RNA. By the same criterion, 5′-capped–Ω′-CAT pseudovirus particles gave 317 times as much CAT as particles containing uncapped CAT mRNA without the Ω′ sequence. No equivalent comparison was possible for the unencapsidated mRNAs, since presumably most were degraded and produced very small yields of CAT. Clearly, our pseudovirus particles uncoat and express the encapsidated mRNA efficiently in plant cells whether or not the cells are considered normal TMV hosts.

To determine whether animal cells could

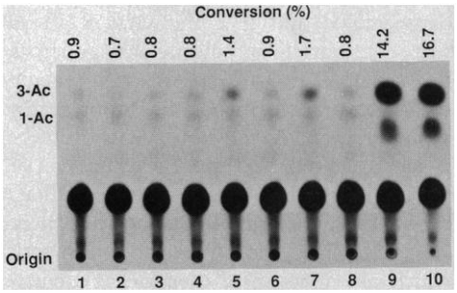

Fig. 4. TLC assay for CAT activity in equal volumes of extract from inoculated pea epidermis. Pea leaves were rubbed with buffer alone (lane 1) or equivalent amounts (on an RNA weight basis) of naked CAT mRNA (lane 2), CAT pseudovirus particles (lane 3), 5′-capped–CAT mRNA (lane 4), 5′-capped–CAT pseudovirus (lane 5), Ω′-CAT mRNA (lane 6), Ω′-CAT pseudovirus (lane 7), 5′-capped–Ω′-CAT mRNA (lane 8), or 5′-capped–Ω′-CAT pseudovirus (lane 9). After 90 minutes, strips of epidermal cells were removed and stored in liquid nitrogen (*7*) before being ground to a frozen powder. Lysed cells were resuspended in 300 μl 0.25*M* tris-HCl, *p*H 7.4, containing 10 m*M* DTT and 2 m*M* leupeptin. Cellular debris was removed and CAT assays were performed on the supernatants as in Fig. 3. As a reference, 0.1 unit of purified CAT was added to a sample of the mock-inoculated extract (lane 10). The TLC plate was autoradiographed for 3 days at room temperature. Spot identities are as in Fig. 3.

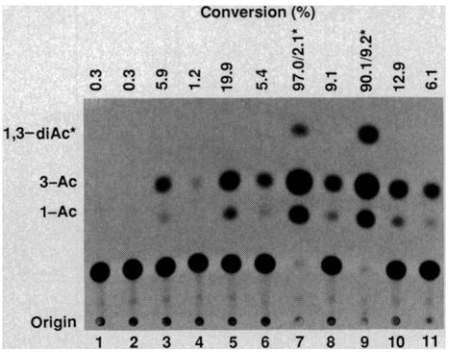

Fig. 5. TLC assay for CAT activity in extracts of microinjected oocytes from *X. laevis* (*16*). Oocytes were injected with water (lane 1); linearized (Bgl II cut) pJII102 DNA to rule out any coupled transcription–translation activity (lane 2); or equivalent amounts (on an RNA weight basis) of naked CAT mRNA (lane 3), CAT pseudovirus particles (lane 4), Ω′-CAT mRNA (lane 5), Ω′-CAT pseudovirus (lane 6), 5′-capped–CAT mRNA (lane 7), 5′-capped–CAT pseudovirus (lane 8), 5′-capped–Ω′-CAT mRNA (lane 9), or 5′-capped–Ω′-CAT pseudovirus (lane 10). After incubation at 20°C for 18 hours, equal numbers of viable oocytes were lysed and centrifuged at 10,000*g* for 5 minutes to sediment yolk material and float off lipids. The intervening liquid was removed in each case for CAT assay as in Fig. 3. As a reference, 0.1 unit of purified CAT was added to a sample of the water-inoculated oocyte extract (lane 11). The TLC plate was autoradiographed for 18 hours at room temperature. Spot identities are as in Fig. 3, except under conditions of very high CAT activity, with 5′-capped naked mRNAs (lanes 7 and 9), where an additional product, 1,3-diacetyl [^{14}C]chloramphenicol (denoted by an asterisk), appeared.

uncoat our pseudovirus particles and express the mRNA, oocytes from *Xenopus laevis* were microinjected (*16*) separately with each CAT mRNA construct or with equivalent amounts of encapsidated CAT mRNAs. CAT activities in the resulting cell extracts are shown in Fig. 5; all assays with pseudovirus-inoculated oocyte extracts were in the linear response range of CAT activity. Unlike tobacco or pea cells, this system responded more efficiently to naked CAT mRNAs than to encapsidated mRNAs (Fig. 5). However, a significant level of pseudovirus particle disassembly and subsequent CAT mRNA expression must have occurred (Fig. 5, lanes 4, 6, 8, and 10). It has been proposed (*17*) that plant virus particles are disassembled and that the viral RNA is released extracellularly during passage across the cellulose walls of plant cells. Events within an amphibian oocyte cannot necessarily be related to the early events of infection of a leaf cell; however, the expression of CAT mRNA in oocytes does suggest that, even in the cytoplasm of an animal cell, there exists suitable and sufficient machinery which can disassemble pseudo-TMV particles. In oocytes (*10*), the presence of a 5′ cap on the CAT mRNA caused a substantial stimulation of CAT expression.

In all our studies described here and elsewhere (*10*), addition of both a 5′ cap and the TMV-leader sequence (Ω′) resulted in maximum stimulation of CAT expression from either naked or packaged mRNA. This observation may have mechanistic implications for the process of cotranslational disassembly (*6–8*).

Our results demonstrate the efficient encapsidation, delivery, and transient in vivo expression of a "foreign" reporter gene transcript within a plant pseudovirus particle vector. A useful feature of this TMV-based system is that encapsidation appears to be largely independent of transcript length or sequence (*9, 18*). This work provides new scope for the design of experiments to examine virological phenomena such as cross-protection (*19*) by delayed or inhibited uncoating of a suitable reporter transcript in a transformed or previously infected plant. The ability to produce transient pulses of a particular mRNA, applied directly to the intact leaf, may be useful for studies on the homeostatic or developmental regulation of plant gene expression, for example, the introduction of early, late, or tissue-specific genes at a novel location. Amplification and constitutive expression would require trans-acting virus replication functions and the relevant cis-acting RNA sequences.

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20. Supported by Diatech Ltd., London. D.E.S. holds a Science and Engineering Research Council Cooperative Award in Science and Engineering postgraduate studentship. We are grateful to P. Watkins and C. Mason for technical assistance, and to K. Plaskitt for the electron micrographs. We thank H. W. Woolhouse, J. W. Davies, R. Hull, and M. Zaitlin for constructive comments on the original text.

22 December 1986; accepted 31 March 1987