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Microinjection analysis of envelope-glycoprotein messenger activities of avian leukosis viral RNAs

(microinjection/oncornavirus/messenger RNA)

DENNIS W. STACEY, VINCENT G. ALLFREY, AND HIDESABURO HANAFUSA

The Rockefeller University, New York, New York 10021

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ABSTRACT **Virion RNA from the avian leukosis virus Rous-associated virus 2 (RAV-2) and poly(A)-containing RNAs from RAV-2-infected chick embryo fibroblasts were microinjected into fibroblasts transformed by the Bryan high-titer strain of Rous sarcoma virus (RSV), which is deficient in viral envelope glycoprotein. Production of infectious RSV following these injections depended upon the viral envelope-messenger activity of the injected RNA. This system constituted a sensitive and rigorous assay system for viral envelope-messenger RNA. It was found that 21S mRNA from RAV-2-infected cells expressed the highest activity, while 35S mRNA expressed comparatively little. In addition, RAV-2 virion RNA expressed little messenger activity. The rate of formation of infectious RSV following 21S mRNA injections reached a peak near 9 hr, which was followed by a rapid decline. Evidence has been obtained that a small fraction of both 35S virion RNA and 35S mRNA from virus-infected cells was encapsulated into virus particles following their injection into virus-producing cells.**

The virions of avian RNA tumor viruses contain two identical, 35–40 S, single-stranded RNA molecules. In avian leukosis viral RNA the *gag* gene, coding for viral group-specific internal proteins, is located near the 5′ terminus (1), while the *env* gene, which codes for viral envelope glycoprotein, is located near the 3′ terminus (2, 3). The genome of the Bryan high-titer strain of Rous sarcoma virus (RSV) contains the *src* gene (whose function induces transformation of infected fibroblasts) in the place of the *env* gene, thus being defective in the formation of infectious progeny (4).

The 35S RNA of RNA tumor viruses contains a methylguanosine cap structure at the 5′ end (5, 6) and poly(A) at the 3′ end (7), and is considered to be the positive strand in the sense that viral RNA functions as messenger RNA within infected cells (8, 9). In cell-free translation systems viral 35S RNA directed the synthesis of high-molecular-weight peptides containing viral group-specific antigenic sites (10, 11). Similar results were obtained following the microinjection of viral RNA into *Xenopus* oocytes (12). There was, however, no evidence for the synthesis of viral envelope proteins in either system with viral RNA as the messenger, indicating that translation initiated only at the 5′ terminus of the viral RNA and proceeded only far enough along the RNA to produce the proteins coded for by 5′-proximal sequences in detectable quantities. In recent hybridization studies with cells infected by the Rous-associated virus 2 (RAV-2), Hayward *et al.* detected *env*-specific sequences in 21S as well as 35S poly(A)-containing molecules (13, *). This raised the possibility that the smaller (21 S) *env*-specific RNA molecules may function as the primary envelope-protein messenger within RAV-2-infected cells. It is known, for example, that a 20–22S virus-specific mRNA is formed in cells infected by murine leukemia virus (14).

Previous studies have demonstrated that the technique of microinjection can be employed to permit translation studies to be carried out within living, cultured cells (15). In this work an attempt was made to use microinjection of RNA to determine the envelope-glycoprotein messenger activities of various size fractions of mRNA from RAV-2-infected cells and of RAV-2 virion RNA. In order to detect the small amount of envelope proteins formed in microinjected cells, an extremely sensitive method had to be employed. We used the complementation of RSV, which is deficient in formation of envelope glycoprotein. When RNA was injected into RSV-transformed cells, infectious virus were released in direct relation to the viral envelope-glycoprotein messenger activity of the injected RNA.

MATERIALS AND METHODS

Microinjection. Microinjection was performed as previously described (15) using micropipettes with outside diameters near 0.5 μm. Injection size was estimated to average 5% of the cell volume as before (15). Prior to injections, all the cells except in a 2-mm^2 area were removed from cover slips. Injected cultures of RSV(−)-transformed cells (for definition see *Cell Culture*) contained 50–70% transformed cells, which were the only cells to receive injections. Injections were made at room temperature into about 500 cells (generally in less than 1 hr) on a coverslip which was then placed in a 35 mm plate and incubated with 2 ml of growth medium at 37°. The entire 2 ml of culture fluid was collected for virus assay every 3 hr for the first 24 hr after injections and at various intervals thereafter.

Cell Culture. Chick embryo fibroblasts (CEF) and quail (*Coturnix japonica*) cell preparation and culture conditions have been described (16, 17). CEF were negative in the endogenous expression of group-specific antigen and chicken helper factor. *RSV(−)-transformed cells* in this paper refers to CEF infected with the Bryan strain of RSV(−) virions in the presence of ultraviolet-light-inactivated Sendai virus (18, 19). RSV(−) virions were produced by RSV(−)-transformed cells and contain no helper virus (19). No virus infectious for CEF (C/E type, which are resistant to infection with subgroup E virus) were ever observed to be released from these RSV(−)-transformed cultures. For each microinjection series, RSV(−)-transformed cells from the same preparation were plated on individual coverslips. RAV-2 and RSV with RAV-2 envelope [RSV(RAV-2)]-infected CEF were prepared by infection, in the presence of DEAE-dextran, of secondary cultures of CEF with approximately 1 infectious unit per cell followed

Abbreviations: RSV, Bryan high-titer strain of Rous sarcoma virus; RAV, Rous-associated virus; CEF, chick embryo fibroblasts; RSV(RAV-2), RSV with RAV-2 envelope; RSV(−), RSV produced by cells lacking chicken helper factor—virions are noninfectious and contain no glycoprotein.

*W. S. Hayward, unpublished data.

by two passages to ensure that all the cells were infected. For virus collection, the second such passage was made into roller bottles from which supernatants were later collected every 3 hr and virus was purified as described before (20).

Infectious RSV production by injected cultures was determined by examining culture fluids for virions capable of producing foci on CEF according to the method described by Rubin (16) except for the addition of DEAE-dextran at the time of infection (17). To eliminate possible contamination of harvested culture fluids with viable cells that could themselves produce foci, the culture fluids were always frozen after routine centrifugation at 900 × *g*, prior to their use in focus assays. In addition, many samples were sonicated for 3 min at 1.0 ampere in a Raytheon sonic oscillator followed by centrifugation at 900 × *g* for 20 min prior to focus assay. To ensure that these procedures would eliminate all cells from supernatants, 1×10^6 RSV(−)-transformed cells were suspended in 2.0 ml of growth medium and treated as above. No cells were observed to survive and produce foci in subsequent assay plates. Therefore, the appearance of even 1 focus-forming unit in supernatants from injected cells is considered significant.

RAV-2 produced by injected cells was detected by subculture of focus assay plates (after the foci had formed) together with 2×10^5 RSV(−)-transformed cells. Thirty-six to forty-eight hours later supernatants from these cultures were assayed for infectious RSV(RAV-2), which would be released in direct proportion to the number of RAV-2-infected cells in the original focus assay plate.

For interference tests, CEF were infected with approximately 1 infectious unit per cell of either RAV-1 or RAV-2, subcultured after 3 days, and infected with RSV-containing samples after 5 days. Chicken antibodies to RAV-1 and RAV-2 were diluted 1:100 into virus samples which were then incubated 30 min at 37° and assayed as usual for infectious RSV. Infectivity for Japanese quail cells was tested in the same way as described for CEF. In each of these analyses, samples of virus released from injected cells were assayed along with duplicate samples of RSV(RAV-1) and RSV(RAV-2), which served as standards to ensure the specificity of the analyses.

RNA Preparation. Messenger RNA was collected from infected cells by treating them with 2 ml per 1×10^7 cells of a solution containing proteinase K at 2 mg/ml (E. M. Laboratories; Elmsford, NY) and 0.1 M NaCl/0.01 M Tris·HCl, pH 7.4/0.01 M EDTA/0.5% sodium dodecyl sulfate for 30 min. Then the RNA was extracted with phenol/chloroform, passed over a poly(U)-Sepharose affinity column, and fractionated by sedimentation on 15–30% sucrose gradients for 7 hr at 40,000 rpm in an SW 40 (Beckman) rotor as previously described (15). [^{3}H]Uridine-labeled ribosomal RNA was run as a standard either in a parallel gradient or as an internal marker added to the mRNA preparation. Prior to sucrose gradient sedimentation, RNA preparations were denatured in 0.01 M Tris·HCl, pH 7.4/0.01 M EDTA/0.2% sodium dodecyl sulfate at 80° for 90 sec. Virion RNA was prepared exactly as mRNA from a pellet of purified virus particles (20) but without affinity chromatography. In preparation for its microinjection, RNA was concentrated by ethanol precipitation and redissolved in a 10% ribonuclease-free sucrose (Schwarz/Mann, Orangeburg, NY) solution.

RESULTS

21S RNA Acts as Primary Envelope Messenger. Poly(A)-containing RNA from RAV-2-infected CEF was separated into size fractions by sucrose gradient sedimentation, and various fractions from the gradient were injected into 200 RSV(−)-transformed cells in order to determine if the injected RNA molecules would direct the synthesis of the envelope proteins needed for the production of infectious RSV particles. Fig. 1 compares the amounts of RSV infectious for CEF released in the first 15 hr following these injections to the size of the injected mRNA. Hybridization studies have indicated the presence of roughly equal amounts of *env*-specific sequences in the 35S and 21S mRNA fractions of RAV-2-infected cells (13, *). In this experiment, however, the 35S mRNA† fraction expressed only little envelope-messenger activity, whereas a strong messenger activity was demonstrated with the 21S–24S mRNA†. It may be argued that greater degradation of the 35S than the 21S RNA might account for the differences in the mRNA activities seen. But greater than 10-fold differences in the envelope-messenger activities of the two size classes of mRNA have consistently been observed. Furthermore, the presence of intact 35S viral RNA molecules in the RNA from appropriate regions of the gradient was demonstrated by their encapsulation to form RAV-2 particles, as will be discussed below.

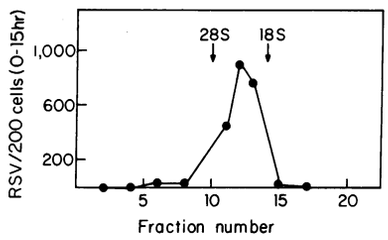

FIG. 1. Envelope-protein messenger activity of various size fractions of mRNA from RAV-2-infected CEF. Poly(A)-containing RNA from RAV-2-infected CEF was fractionated by sucrose gradient sedimentation. The RNA of each fraction (0.5 ml) analyzed was concentrated to 5 μl and aliquots (approximately 0.5×10^{-10} ml) were injected into each of 200 RSV(−)-transformed cells. The number of infectious RSV released within the first 15 hr following injections was then determined. The solution injected for fraction 12 contained 0.75 mg/ml of RNA, while fraction 6 contained 0.26 mg/ml of RNA. As evidence that the above gradient contained biologically active 35S mRNA molecules, preliminary results indicated that RNA of fraction 6, but not of fractions 9 or 12, expressed viral polymerase messenger activity. This determination was made following injections of mRNA into CEF transformed by the polymerase-deficient RSVα (unpublished results).

The time course of production of infectious RSV from transformed cells following 21S mRNA injection is shown in Fig. 2*A*. Infectious RSV were released starting 3 hr following injection with a maximal rate of release near 9 hr. This peak was followed by a rapid decline in the rate of RSV release to a constant level which persisted for the 51 hr duration of this experiment. No RAV-2, however, was detected within the first 24 hr following injection.

The virus released between 6 and 15 hr following injection was analyzed to determine its subgroup. The focus-forming virus was interfered with by RAV-2 (but not RAV-1) and neutralized by chicken anti-RAV-2 serum (but not anti-RAV-1 serum). No foci were observed to form on quail cells. These results indicated that the virus released following mRNA injections into RSV(−)-transformed cells were of the same subgroup as RAV-2, and yet were free of RAV-2 virus particles. The virus released thus appear to constitute a pure population of RSV(RAV-2).

The relationship between mRNA concentration and the re-

† The term *mRNA* will be used hereafter to denote poly(A)-containing RNA from virus-infected CEF while *virion RNA* will denote RNA prepared from virus particles. mRNA in the 21S–24S region of sucrose gradients will be referred to as *21S mRNA*.

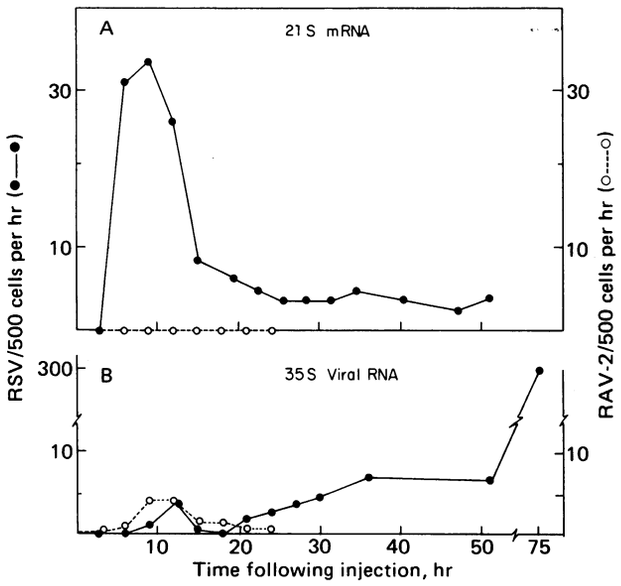

FIG. 2. Time course of release of infectious virus following microinjection of (*A*) 21S mRNA and (*B*) 35S virion RNA into RSV(−)-transformed cells. 21S poly(A)-containing RNA from RAV-2-infected cells was concentrated to 0.75 mg/ml, while 35S RAV-2 virion RNA was concentrated to 7 mg/ml. Each was injected into 500 RSV(−)-transformed cells. The titers of infectious RSV and RAV-2 were determined at various times thereafter. In the case of RAV-2, the titers indicate relative numbers of virus released.

lease of infectious RSV was studied by injecting three different concentrations of 21S mRNA into 300 RSV(−)-transformed cells. As shown in Fig. 3, within the limitations of the microinjection technique employed in this work, the total numbers of infectious RSV produced within 24 hr were proportional to the injected mRNA concentrations. In addition, the time course of virus release was similar following injection of each RNA concentration.

Low Messenger RNA Activity of 35S Virion RNA. The foregoing discussion has involved studies of the envelope-

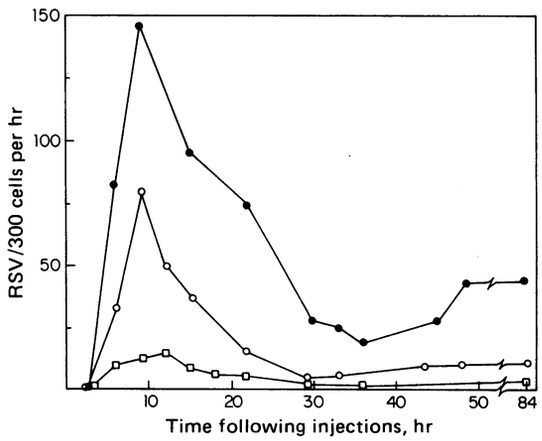

FIG. 3. The relationship between mRNA concentration and infectious RSV release. 21S poly(A)-containing RNA from RAV-2-infected cells was prepared in three concentrations: 0.78 mg/ml (●), 0.30 mg/ml (○), and 0.08 mg/ml (□). Each solution was injected into 300 RSV(−)-transformed cells and infectious RSV released at various times thereafter was determined. (The culture receiving the 0.78 mg/ml of solution became superinfected by RAV-2 following injection, indicating that the injected RNA contained a low concentration of contaminating 35S mRNA.)

Table 1. Envelope-messenger activity of 35S RAV-2 virion RNA*

RNA preparation	Concentration of RNA, mg/ml	RSV released in 1–24 hr†	RAV-2 released in 1–24 hr‡	Superinfection of injected cultures §
A	20	12	0	+
	7	26	14	+
	3	18	4	+
	1	13	0	+
B¶	3	112	ND	+
	0.8	60	ND	+
	0.2	4	ND	ND

ND, not determined.

* Various concentrations of two independent RAV-2 35S virion RNA preparations were injected into 500 RSV(−)-transformed cells each. Culture fluids were collected every 3 hr thereafter and analyzed for the amounts of infectious RSV and RAV-2 as described in *Materials and Methods*.

† Focus-forming units on CEF.

‡ Relative amounts of RAV-2 are expressed here as focus-forming units of RSV(RAV-2) produced after addition of the supernatants of injected cell cultures to RSV(−)-transformed cells.

§ Injected RSV(−)-transformed cell cultures were considered to be superinfected if there were large increases in the amounts of infectious RSV released after 24 hr postinjection.

¶ Virus purification for preparation B was performed at room temperature, resulting in increased degradation of viral RNA.

messenger activities of mRNA fractions obtained from RAV-2-infected CEF. 35S RAV-2 virion RNA should also possess the messenger sequences necessary to direct envelope-protein synthesis. The virion RNA was, therefore, injected into RSV(−)-transformed cells to determine if it would function as envelope messenger. Table 1 summarizes the results of two such experiments. Two conclusions are apparent from these results. First, virion RNA serves inefficiently as an envelope messenger within RSV(−)-transformed cells. Relatively few infectious RSV were released even following injections of highly concentrated RAV-2 virion RNA (up to 20 mg/ml). When injection conditions were chosen to produce virion RNA concentrations (0.2 mg/ml) within injected cells comparable to those normally found in virus-infected cells, almost no infectious RSV were produced. Second, separate preparations of virion RNA differed greatly in their envelope-messenger activities. Preparation B in Table 1 expressed approximately 6 times the activity of preparation A. It is interesting to note that 70% of preparation A RNA was in the 35S RNA peak, while preparation B was degraded to a greater extent and contained only approximately 30% of its RNA in the 35S RNA fraction. While only the 35S fractions of each preparation were injected for this experiment, the increased activity of preparation B may have resulted from greater contamination with smaller fragments of viral RNA. This hypothesis is supported by the observation that 21S fragments of RAV-2 virion RNA of preparation B (5 mg/ml) promoted the production of 1680 infectious RSV particles in the first 24 hr following injection into 500 RSV(−)-transformed cells. It is in fact possible, in view of this observation, that all the messenger activity expressed by injected 35S virion RNA preparations resulted from contaminating, partially degraded RNA.

Virus Encapsulation of Injected Viral RNA. Fig. 2*B* presents the time course of release of virus from RSV(−)-transformed cells following injections of 35S RAV-2 virion RNA

Table 2. Formation of infectious RSV by injection of 35S mRNA obtained from RSV-transformed cells into RAV-2-infected cells*

RNA donor cell type	Concentration of RNA, mg/ml	No. of cells injected	Infectious RSV released between hr § 0–2	2–6	6–10	10–14
RSV(RAV-2)†	1.2	175	4	2	0	0
RSV(−)‡	5.7	100	1	2	2	0
RSV(−)‡	0.8	500	—	3	0	0

* 35S mRNA from donor cells was microinjected into RAV-2-infected cells. Production of transforming virus was then determined by focus assay on CEF.

† RNA extracted from these RSV(RAV-2)-infected cells was not denatured prior to fractionation on a sucrose gradient to avoid the possible contamination with RNA derived from the 70S RNA of mature virions.

‡ Two different concentrations were made of the same mRNA preparation extracted from RSV(−)-transformed cells.

§ No transforming virus were observed after 14 hr following any of these injections.

(preparation A). There was a small peak of infectious RSV production at 15 hr followed by subsequent gradual then sharp increases at 25 and 50 hr, respectively. The production of large numbers of infectious RSV at later times following virion RNA injections indicated that the injected cells had been superinfected by a helper virus. As one of the mechanisms for helper virus production, it is possible that RAV-2 particles were produced by encapsulation of injected RAV-2 virion RNA into a viral particle released by RSV(−)-transformed cells and rendered infectious by the low level of envelope-messenger activity expressed by the viral RNA preparation. This idea is supported by the fact that infectious RAV-2 particles were released within the first 24 hr following injections of RAV-2 virion RNA into RSV(−)-transformed cells (Table 1, Fig. 2*B*). Virus released at this early time would have to be produced by the direct encapsulation of injected RNA (21). This early RAV-2 production was observed only in transformed cells injected with highly concentrated virion 35S RNA. But cells always became superinfected by RAV-2 particles following injections of RAV-2 virion RNA, even in the absence of observable early release of infectious RAV-2 virus.

Similar observations were made following injections of 35S mRNA. No infectious RAV-2 particles were detected within 24 hr following injections of mRNA. But a gradual increase in RSV production beginning 30 hr following injections was consistently observed, which must indicate RAV-2 formation as described above.

The direct encapsulation of injected virus-specific mRNA into viral particles was further exemplified by the formation of infectious RSV following injection of 35S mRNA from RSV(−)-transformed cells or RSV(RAV-2)-infected cells into RAV-2-producing cells. Encapsulation of injected RSV mRNA resulted in the formation of transforming virus. As shown in Table 2, few transforming particles were observed. These figures were significant, however, because absolutely no RSV infectious for CEF was observed without injection. The release of small numbers of infectious RSV obviously indicates that the encapsulation did not take place frequently. It seemed characteristic for the encapsulation that the formation of infectious particles occurred within a relatively short time period following injection.

DISCUSSION

These studies demonstrate that messenger RNA for RAV-2 envelope glycoprotein can be translated following microinjection into RSV(−)-transformed cells. The fact that the envelope protein synthesized can be properly modified and positioned within the injected cell and budding virus to render the virus infectious, with the subgroup specificity of RAV-2, indicates that the mRNA is translated with high fidelity. In addition, the injected mRNA must be translated within a system that closely approximates that existing in normal virus-infected cells. Injected mRNA must compete with endogenous mRNA to be translated. In this system, artifacts associated with cell-free translation systems (e.g., cellular disruption and dilution of cytoplasmic factors) are avoided.

Of the various size classes of poly(A)-containing RNA obtained from RAV-2-infected cells, 21–24S RNA expressed the greatest degree of messenger activity, while the 35S fraction expressed only about 3% as much. The results described in this work establish that 21–24S mRNA contains the bulk of messenger activity for viral envelope protein within RAV-2-infected cells. It is not entirely conclusive, however, whether either 35S mRNA or intact 35S virion RNA expresses messenger activity for envelope glycoprotein because these RNAs could have been sufficiently contaminated by either 21S mRNA or fragmented virion RNA to account for the activity seen. 35S virion RNA does, however, serve as a messenger for the synthesis of viral core proteins in a cell-free translation system or following injection into *Xenopus* oocytes (10, 11). In addition, our preliminary evidence suggests that within RSVα-transformed cells positive for chicken helper factor (22), both the 35S mRNA and virion RNAs function as messengers for viral polymerase, while 21S mRNA does not.

Because these 35S RNAs did not express high levels of envelope-messenger activity, it is apparent that neither was readily processed within the cytoplasm of injected cells to produce smaller, more active messenger molecules. The origin of the 21S envelope-messenger RNA molecules is not known. It might result from the nuclear cleavage of larger RNA molecules containing other viral genes, or independent transcription of the envelope gene.

Time course studies following mRNA injections consistently revealed a 3 hr lag between injection and the onset of infectious virus production. This delay was not the result of injection-induced trauma, because infectious RSV were released within 2 hr following injection of 35S mRNA from RSV(−)-transformed cells into RAV-2-infected cells. The rapid decline in the rate of virus release after a peak generally about 9 hr after injection suggests that the envelope messenger RNA has a half-life within RSV(−)-transformed cells of near 10 hr.

The highest rate of RSV release in these studies was 0.5 infectious RSV per injected cell per hr, observed following injections of approximately 0.5×10^{-10} ml of a 0.75 mg/ml preparation of 21S mRNA from RAV-2-infected cells. These injections should provide approximately a 3-fold increase of 21S mRNA sequences over that normally attained by virus-infected cells. The efficiency of virus release following these injections was approximately one-half that observed in RSV(−)-transformed cells superinfected with intact RAV-2, suggesting that injected envelope mRNA was translated on the order of one-sixth as efficiently as endogenous mRNA. A pulse of exogenous mRNA, however, would not be expected to produce levels of virus envelope production as high as would equivalent steady-state mRNA concentrations in virus-infected cells, particularly when a 3-hr lag is interposed between synthesis of the envelope and its biological expression. A further compli-

cation is that because injected cells were surrounded by cells that could adsorb virus particles, many of the RSV particles produced by injected cells may have been adsorbed by adjacent cells and thus avoided detection as infectious particles released into the medium. These considerations suggest that injected mRNA is likely to be more active than is indicated by the rate of RSV release following microinjection.

When three different concentrations of 21S mRNA were injected, the numbers of infectious RSV released were roughly proportional to the concentrations of injected RNA. In addition the kinetics of release were similar for each mRNA concentration tested. These observations indicate that the limiting factor in the RSV production in this system was the availability of envelope glycoprotein, even when the rate of release was close to 50% of that observed in normally infected cells. They also suggest that even the injection of the highly concentrated mRNA preparations did not overly saturate the translational capacity of the injected cells.

With highly concentrated 35S virion RNA, RAV-2 particles were released from the injected cells. Because the cells that received the RNA are producing noninfectious RSV(−) particles, virus-coded RNA-dependent DNA polymerase (reverse transcriptase) and viral structural proteins other than the envelope proteins are continuously synthesized in these cells. Two possibilities are conceivable for the formation of infectious RAV-2. One is the encapsulation of injected RAV-2 RNA into particles with RSV-coded enzyme and core proteins and the envelope protein translated from 35S RNA (or from envelope-messenger sequences derived from 35S virion RNA). The second possibility is the synthesis of a RAV-2 provirus with RSV-coded enzyme using the injected 35S RNA as a template within the injected cells; once the provirus is formed and integrated, the cell would produce infectious RAV-2. If, however, the second mechanism is involved in the formation of RAV-2, its release would take a longer time than observed (21). Further, a cell containing an integrated provirus would continuously produce RAV-2. Thus, the observed results showing that the early RAV-2 production declined after a short burst do not support this second possibility. The direct encapsulation is thus considered more likely as a mechanism of RAV-2 formation. However, the provirus formation in injected cells is theoretically possible, and one cannot exclude its possible occurrence in some injected cells.

Encapsulation was not observed in early hours following injection of low concentrations of virion RNA or 35S mRNA, but was apparent from the fact that cultures injected with these RNAs subsequently became the producers of high titers of RSV and RAV-2. The variable time periods required for this rise of RSV and RAV-2 formation suggest that the spread of virus is not always efficient. This may well be due to the low content of envelope protein in the first encapsulated RAV-2 particles and thus its spread may depend on the cell-to-cell transfer rather than the spread by infection.

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