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Messenger Activity of Virion RNA for Avian Leukosis Viral Envelope Glycoprotein

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An intracellular assay for viral envelope glycoprotein (*env*) messenger was employed to analyze the RNA from virus particles of Rous-associated virus type 2. For this assay RNA was microinjected into cells infected by the *env*-deficient Bryan strain of Rous sarcoma virus [RSV(−) cells]. Only when the injected RNA could be translated by the recipient cells to produce viral envelope glycoprotein was the *env* deficiency of the RSV(−) cells complemented, enabling them to release focus-forming virus. RNA in a 21S size fraction from the Rous-associated virus particle promoted the release of numerous focus-forming virus from RSV(−) cells, whereas the major 35S virion RNA species was inactive. The *env* messenger activity sedimented as a sharp peak with high specific activity. RNase T1-generated fragments of virion 35S RNA were unable to promote the release of infectious virus from RSV(−) cells. Consequently, the active molecule was most likely to be *env* messenger which had been encapsulated by the virus particle from the cytoplasm of infected cells. Approximately 95% of the *env* messenger within the virion was associated with the virion high-molecular-weight RNA complex. The temperature required to dissociate *env* messenger from the high-molecular-weight complex was indistinguishable from the temperature required to disrupt the complex itself. Virion high-molecular-weight RNA that was associated with *env* messenger sedimented slightly more rapidly than the bulk virion RNA; this was the strongest evidence that the 21S messenger had been encapsulated directly from the infected cells. These data are considered along with a related observation [concerning the prolonged expression of *env* messenger after injection into RSV(−) cells] to raise the possibility that virus-encapsulated *env* messenger can become expressed within subsequently infected cells.

Retrovirus particles contain two 30–40S RNA molecules, each of which encodes the entire virus genome (1, 4, 17). These associate together within the virion to form a high-molecular-weight complex (10, 23). In mammalian retroviruses this complex is stabilized by base pairing between the two RNA subunits at their 5′ termini (3). This type of association has not yet been observed in avian leukosis viruses, although it is likely that RNA structure within mammalian and avian viruses is similar (2).

Cells infected by an avian leukosis virus contain two major virus-specific RNA species. One is similar if not identical to the 35S molecule within the virus particle and is believed to function as messenger for viral core proteins and polymerase (19, 21, 31). The other molecule sediments at 21S and contains primarily the nucleotide sequences of the envelope glycoprotein (*env*) gene (14, 33), which is located in the 3′ half of the 35S molecule (16, 32). The 5′ termini of both cellular RNA molecules contain identical nucleotide sequences (18, 33), suggesting that the 21S molecule is the product of RNA processing.

Both the 21S and 35S cellular RNA species contain the *env* gene; the technique of microinjection was used to demonstrate, however, that only the 21S molecule could serve efficiently within the cell as messenger for envelope glycoprotein. Various size fractions of mRNA from cells infected by Rous-associated virus type 2 (RAV-2) were microinjected into the cytoplasm of cells transformed by the *env*-deficient Bryan strain of Rous sarcoma virus [RSV(−) cells]. Sarcoma virus particles released by RSV(−) cells were not infectious because they lacked envelope glycoprotein (12). When RNA that could function as *env* messenger was injected into the cytoplasm of RSV(−) cells, the *env* deficiency was complemented and transforming virus were released. Only the 21–24S fractions of mRNA from RAV-2-infected cells efficiently promoted the release of transforming virus from RSV(−) cells (27).

RNA from RAV-2 virus particles was also

analyzed. The predominant 35S virion RNA species was inactive as *env* messenger following injection into the cytoplasm of RSV(−) cells, but upon injection into their nuclei, virion 35S RNA promoted the release of numerous infectious virus from RSV(−) cells. The 35S virion RNA had apparently been converted within the nuclei to *env* messenger (28), suggesting that 21S *env* messenger is produced by nuclear processing.

In contrast to 35S virion RNA, subgenomic RNA from the virus particle functioned directly as *env* messenger upon cytoplasmic injection into RSV(−) cells (27). Evidence will be presented here to suggest that the active molecule was 21S *env* messenger encapsulated by the virus particle directly from the infected cell. The possible implications of this observation are discussed.

MATERIALS AND METHODS

Cell culture and microinjection. Microinjection techniques have been described elsewhere (9, 26). From 200 to 400 RSV(−) cells were injected for each determination. Culture fluids were collected from injected cells at various intervals for up to 15 h after injection. The preparation and handling of RSV(−) cells, focus assay, and antibody neutralization determination have been described (27). All chicken embryo fibroblasts used were shown to express no endogenous viral functions and to be susceptible to subgroups A and B but not subgroup E viruses (C/E cells). The titer of RAV-2 virus was determined by end-point dilution as follows. Chicken embryo fibroblasts were infected by various dilutions of the sample (in the presence of DEAE-dextran) and allowed to grow to confluence before subculture with 5×10^5 RSV(−) cells. Two days after subculture, fluids were collected from the plates and assayed for focus-forming virus, which indicated the presence of helper virus in the original inoculum. Numerous determinations were made at each dilution, and the proportion of helper virus-containing plates at each dilution was used to determine the RAV-2 titer.

RNA handling. RNA was prepared from RAV-2 (or RAV-7) virus particles collected at 2- to 3-h intervals. For comparison, some virus were collected at up to 12-h intervals. Virus were purified by isopycnic centrifugation on 20 to 50% sucrose gradients (in 0.15 M NaCl–10 mM Tris-hydrochloride, pH 7.4) for 12 h at 25,000 rpm in a Beckman SW27 rotor. RNA was extracted from the purified virus by treatment with proteinase K at 2 mg/ml (E.M. Laboratories, Elmsford, N.Y.) and 0.5% sodium dodecyl sulfate for 20 min at room temperature, followed by extraction of the reaction mixture with phenol–chloroform (1:1) as described (27). Virion RNA was labeled by adding [^{3}H]uridine (New England Nuclear Corp., Boston, Mass.) to infected cells 3 h before virus collection. Velocity sedimentation of RNA was performed on 15 to 30% sucrose gradients as described (27) for 6.5 h for denatured and 3.0 to 3.5 h for undenatured virion RNA. Ribosomal RNA size markers were sedimented in parallel gradients. RNA was prepared for microinjection by pelleting from an ethanol precipitate for 30 min at 30,000 rpm in a Beckman SW50.1 rotor. The RNA pellet was similarly washed with 70% ethanol, then lyophilized and dissolved in 0.15 M KCl. RNA concentrations ranged from 0.2 to 4.0 mg/ml.

Denaturation of virion RNA was performed immediately before velocity sedimentation in 0.05 M NaCl–10 mM Tris-hydrochloride–1 mM EDTA–0.2% sodium dodecyl sulfate (pH 7.4) (0.05 M ETS) at 85°C for 90 s followed by rapid cooling to room temperature. For partial denaturation, purified 60–70S virion RNA was dissolved in 0.1 M ETS and heated to 50 to 60°C for 2.5 min, followed by rapid cooling and velocity sedimentation. The messenger activity of virion high-molecular-weight RNA was compared with denatured RNA by first dissolving 60–70S RNA in 0.15 M KCl. Part of the RNA sample was injected into RSV(−) cells, and the rest was kept at 0°C (approximately 20 min). After injection of undenatured RNA, the unused RNA was denatured at 100°C for 75 s, rapidly cooled, and injected into duplicate RSV(−) cells.

RNase T1 (Calbiochem, Somerville, N.J.) was used to fragment 35S RAV-2 virion RNA that had been purified twice by velocity sedimentation and analyzed as described in the text. The RNA was dissolved in 0.15 M NaCl–0.05 M Tris-hydrochloride (pH 7.4)–1 mM EDTA. RNase T1 at 10^{-3} U/ml was added, and the mixture was incubated at 37°C for 3 to 7 min. The reaction was stopped by the addition of proteinase K (2 mg/ml) and sodium dodecyl sulfate (0.5%), and the mixture was incubated for 10 min at room temperature before extraction with phenol–chloroform. To produce fragments of varying sizes the following protocol was followed. Purified 35S virion RNA was dissolved and divided into three equal volumes. RNase T1 was then added at the concentration of 5×10^{-3}, 1×10^{-2}, and 5×10^{-2} U/ml. The three samples were incubated for 3 min at 37°C, treated with proteinase K and sodium dodecyl sulfate, and combined prior to phenol extraction. Polyadenylic acid-containing fragments were bound to oligodeoxythymidylic acid-cellulose (Collaborative Research, Waltham, Mass.) in 0.5 M NaCl buffer and eluted in the same without NaCl (10 mM Tris-hydrochloride–1 mM EDTA, pH 7.4). Fragments were denatured as described above before velocity sedimentation.

RESULTS

Size of virion *env* messenger. To determine the size of *env* messenger within the virus particle, heat-denatured RNA from purified RAV-2 virus particles was fractionated by velocity sedimentation. A constant proportion of the RNA in each size fraction of the gradient, from 12S to 35S, was then microinjected into the cytoplasm of 400 RSV(−) cells. The major 35S RNA species failed to promote the release of any focus-forming units (FFU) from the injected cells, indicating that it had not functioned as messenger for viral envelope glycoprotein to

complement the *env* deficiency of the RSV(−) cells. RNA in the 21S size fraction, on the other hand, promoted the release of numerous FFU from the injected cells (Fig. 1). The data indicated that the active virion *env* messenger constituted a uniform species with sedimentation characteristics similar to cellular *env* messenger. [The number of FFU released after injections into RSV(−) cells is directly proportional to the *env* messenger activity of the RNA injected (27)].

The virion 21S RNA had a high specific activity as *env* messenger (64 FFU/pg of injected RNA, released within 9 h after injection) in relation to the most active 21S mRNA fractions prepared from RAV-2-infected cells (9.6 FFU/pg [27]). In similar experiments, 21S virion RNA from another leukosis virus, RAV-7, expressed messenger activity for envelope glycoprotein, although at a much lower specific activity than observed with RAV-2. Finally, the infectious virus released after injections of RAV-2 virion RNA possessed the antibody neutralization characteristics of RAV-2 (data not shown); this indicates that the virion RNA had faithfully directed synthesis of the envelope glycoprotein molecule.

Inactivity of RNA fragments. The 21S virion messenger might be either *env* messenger encapsulated directly from the infected cell or a product of 35S RNA cleavage within the virus particle.The specific messenger activity (and in many cases the total messenger activity) of 21S RNA, however, was consistently greater in RNA prepared from virus harvested at short intervals (2 h). The high activity of this RNA, which would have undergone a minimum of degradation within the virus particle, argued against generation of the active molecule by cleavage within the virion.

Further evidence against generation of the *env* messenger within the virion came from the fact that fragments of virion RNA were inactive as *env* messenger. 35S RAV-2 virion RNA was purified twice by velocity sedimentation to remove contaminating 21S *env* messenger. The purified 35S RNA was injected into the cytoplasm of RSV(−) cells to verify that no detectable *env* messenger activity remained. The RNA was next injected into the nuclei of RSV(−) cells to verify that it retained activity as nuclear precursor to *env* messenger. The 35S RNA was then fragmented by RNase T1 as described in Materials and Methods. Fragments containing polyadenylic acid, and consequently the *env* gene, were selected on oligodeoxythymidylic acid-cellulose and fractionated by velocity sedimentation. RNA in size fractions corresponding to 24S, 21S, and 18S (Fig. 2) was separately concentrated to 1.2 mg/ml and injected into 300, 600, and 300 RSV(−) cells, respectively. In no case were any FFU released from these injected cells (Table 1).

As a positive control, 35S RNA that had escaped hydrolysis by RNase T1 (Fig. 2) was concentrated to 0.75 mg/ml and injected into the nuclei of 300 RSV(−) cells; 610 infectious virus were released. Since this 35S RNA was obtained from the same hydrolysis reaction and the same sucrose gradient as the fragments described

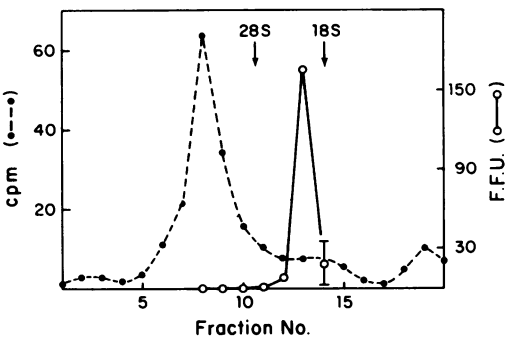

FIG. 1. *Size of env messenger in denatured virion RNA. Total ^{3}H-labeled RNA from purified RAV-2 virus particles was denatured and fractionated by velocity sedimentation. A given volume of each fraction, from 8 to 14, was concentrated to 2.5 μl and microinjected into 400 RSV(−) cells. env messenger activity sedimented as a sharp, 21S peak as indicated by the number of FFU released; the major 35S virion RNA species was inactive. Fewer than five RAV-2 virus were released after any injection. The RNA injected for fraction 13 was 0.2 mg/ml.*

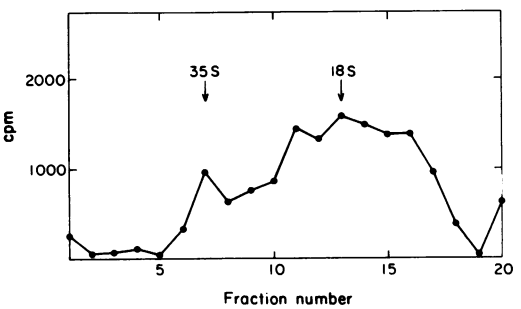

FIG. 2. *Size distribution of virion RNA fragments. 35S RNA from purified, [^{3}H]uridine-labeled RAV-2 virus particles was twice purified and analyzed for biological activity as described in the text. The RNA was then divided into three parts and treated with varying concentrations of RNase T1 to yield a broad range of fragment sizes (see Materials and Methods). Polyadenylic acid-containing fragments were then selected on oligodeoxythymidylic acid-cellulose and fractionated by velocity sedimentation. RNA in the small peak at 35S represents virion RNA that had escaped hydrolysis.*

TABLE 1. *Inactivity of RNA fragments as env messenger*

Fragment size	Prepn[a]	RNA concn (mg/ml)	No. of injections	Injection site[b]	No. of virus released
18S	A	1.2	300	C	0
21S	A	1.2	600	C	0
24S	A	1.0	300	C	0
35S[c]	A	0.75	300	N	610
16–24S	B	1.2	400	C	0
15–25S	C	0.5	400	C	0
15–26S	D	2.0	350	C	0
21S	E	0.5	300	C	0

[a] Purified 35S RAV-2 virion RNA was hydrolyzed by varying concentrations of RNase T1 (preparation A; see legend Fig. 2), or by 10^{-3} U of RNase T1 per ml (preparations B and C). Fragments in preparations A and B were passed over oligodeoxythymidylic acid-cellulose. All RNA preparations were fractionated by velocity sedimentation. If only one fraction was used as the source for injected RNA, the sedimentation value of that fraction is listed. If more than one fraction was used as the source of injected RNA, the inclusive sedimentation values are given. Preparations D and E were hydrolyzed by homogenates of HeLa cells in 0.1 M KCl–10 mM Tris-hydrochloride (pH 7.4).

[b] Injections were performed into the nucleus (N) or cytoplasm (C) of RSV(−) cells.

[c] 35S RNA was that which had escaped hydrolysis by RNase T1.

above, the inactivity of the fragments must not have resulted from handling of the RNA. It is calculated that if as many 35S RNA molecules had been injected into nuclei as there were 21S RNA fragment molecules injected into the cytoplasm (in the experiment described above) approximately 10^4 infectious virus would have been released. The specific activity of the 35S RNA injected into the nucleus was high (100 FFU/pg) in comparison to 21S *env* mRNA preparations within the cytoplasm (despite the fact that only about one-third of the 35S molecule can be converted into *env* messenger). In other experiments, RNase T1 fragments of 35S RAV-2 virion RNA in varying concentrations and size ranges, and without selection on oligodeoxythymidylic acid-cellulose, were inactive as *env* messenger (Table 1).

A similar result has been reported in which mild alkaline hydrolysis of 35S RAV-2 virion RNA was shown to be ineffective in generating *env* messenger (28). In addition, total cytoplasmic nucleases from HeLa cells were used to cleave 35S virion RNA without the generation of detectable *env* messenger (Table 1).

Although random in vitro fragmentation of 35S RAV-2 virion RNA by either chemical or enzymatic techniques did not generate RNA that could function as *env* messenger within RSV(−) cells, it is possible that specific cleavage of 35S RNA within the virion could generate *env* messenger. Even specific fragments, however, would have to function actively without the 5′ leader sequence normally found in *env* messenger (18, 33), and would appear within the cell without a 5′ cap structure.

env messenger in the high-molecular-weight virion RNA complex. Undenatured RNA from RAV-2 virus particles was fractionated by velocity sedimentation to display the virion high-molecular-weight 60–70S RNA. A constant proportion of RNA in each size fraction from 15S to 70S was microinjected into RSV(−) cells. The release of infectious virus indicated the *env* messenger content of each fraction. Approximately 95% of the *env* messenger activity was associated with 60–70S RNA. A small amount of free 21S messenger was observed, but no activity was associated with 35S RNA (Fig. 3). The *env* messenger was stably associated with high-molecular-weight RNA, because ethanol precipitation and refractionation of the 60–70S RNA did not promote the release of 21S *env* messenger.

Thermal dissociation of *env* messenger from virion RNA. To characterize the bond stabilizing 21S *env* messenger within the virion high-molecular-weight complex, its thermal stability was determined. RAV-2 virion 60–70S RNA was collected from a sedimentation gradient, heated to various temperatures (from 50 to 60°C in 0.1 M NaCl), and refractionated by velocity sedimentation. As high-molecular-

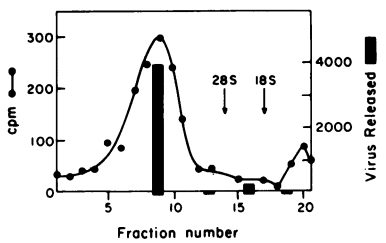

FIG. 3. *Sedimentation characteristics of env messenger in undenatured virion RNA. Undenatured RNA from purified RAV-2 virus particles was fractionated by velocity sedimentation. Every second fraction from the resulting gradient was combined with the one just before it, and a given volume of the combined fractions was then concentrated to 4 μl, denatured, and microinjected into 250 RSV(−) cells. ^{3}H-labeled RNA in the high-molecular-weight region (fractions 6–11) promoted the release of approximately 20-fold more infectious virus than that in the 21S region (fractions 14–17). No virus were released upon injection of RNA in the 35S region (fractions 12–13) or below 18S (fractions 18–19). The RNA injected for fraction 8–9 was 4 mg/ml.*

weight RNA is denatured, its sedimentation value shifts abruptly from 60–70S to 35S (6), so that the ratio of RNA in these two regions of the sedimentation profile indicated the extent of denaturation of the RNA complex. The extent of release of *env* messenger from high-molecular-weight RNA was determined by microinjection. RNA in three regions of the gradient (60–70S, 35S, and 21S; see Fig. 4) was microinjected into RSV(−) cells. The number of FFU subsequently released indicated the relative proportion of *env* messenger in each gradient region.

The thermal stability of the *env* messenger within the virion RNA complex was shown to be nearly identical to that of the complex itself. At 50°C the virion RNA complex was stable and over 95% of the *env* messenger remained associated with the 60–70S RNA. RNA treated at 58°C was almost totally disrupted, and 95% of the RNA sedimented at 35S or less. In this gradient all *env* messenger sedimented in the 21S region. When the virion RNA was treated at intermediate temperatures, the RNA ratio (60–70S RNA:35S RNA) roughly equaled the ratio of messenger activity (60–70S activity:21S activity). Figure 4 presents the profile of high-molecular-weight RNA heated to 54°C. Approximately 30% of the RNA had been converted from 60–70S to 35S, and 22% of the messenger activity sedimented at 21S. No activity was reproducibly identified with the 35S regions of these gradients, indicating that there were no intermediates in the conversion of high-molecular-weight messenger to free 21S messenger.

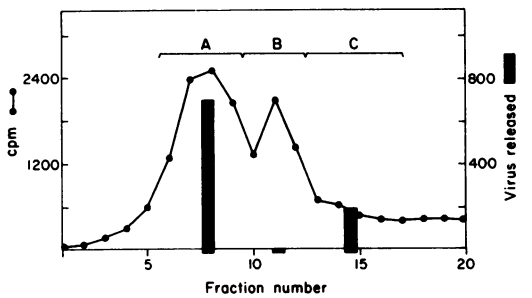

FIG. 4. *Thermal stability of env messenger in the virion RNA complex. 60–70S RAV-2 virion RNA (labeled with [³H]uridine) was heated to 54°C prior to fractionation by velocity sedimentation. The resulting RNA profile indicated that only part of the high-molecular-weight RNA had been denatured. A constant proportion of RNA in the high-molecular-weight (A), 35S (B), and 21S (C) regions of the gradient (as indicated by brackets) was then microinjected into 350 RSV(−) cells. env messenger had been only partially dissociated from the virion high-molecular-weight complex, as indicated by virus released from injected cells.*

These results indicate that the bond holding *env* messenger within the virion RNA complex was similar if not identical to the bond that stabilized the virion complex itself. This bond might have involved the 5′ terminus of the *env* messenger, which was identical to that of the 35S virion RNA subunits (and in mammalian virus is known to play a central role in stabilization of the virion RNA complex [2, 3]). Alternately, the 21S *env* messenger might have been physically entrapped by the high-molecular-weight complex and released only as the complex was disrupted. In either case it is apparent that the *env* messenger and the virion RNA complex were in intimate contact.

Size of the *env* messenger-containing virion RNA complex. The association of 21S *env* messenger with the virion high-molecular-weight RNA complex is likely to alter the sedimentation characteristics of the latter. To test this possibility, undenatured RAV-2 virion RNA was fractionated by velocity sedimentation. High-molecular-weight RNA in the most rapidly and the most slowly sedimenting regions of the resulting profile were then separately refractionated by a second sedimentation step (Fig. 5). Finally, the most rapidly and the most slowly sedimenting regions of the second high-molecular-weight RNA profiles were prepared in equal concentrations (approximately 1.5 mg/ml) and each was injected into 400 RSV(−) cells (Fig. 5). The rapidly sedimenting RNA contained proportionately more *env* messenger and promoted the release of 192 FFU, whereas the slowly sedimenting RNA promoted the release of only 6 FFU.

The factors that influence sedimentation characteristics of virion high-molecular-weight RNA are not known, but it is likely that more rapidly sedimenting RNA complexes contain more RNA. On this basis the association of *env* messenger with rapidly sedimenting virion RNA complexes constitutes strong evidence that the active RNA did not arise by cleavage within the virus particle (since cleavage could not increase the RNA content of the virion high-molecular-weight complex).

Translation of high-molecular-weight virion RNA. Determination was finally made of the availability for translation of *env* messenger RNA within the virion high-molecular-weight complex. 60–70S RAV-2 virion RNA was microinjected into RSV(−) cells either without or just after denaturation (100°C for 75 s in 0.15 M KCl). At best, denaturation resulted in only a three- to fivefold increase in the number of infectious virus released after injections of the

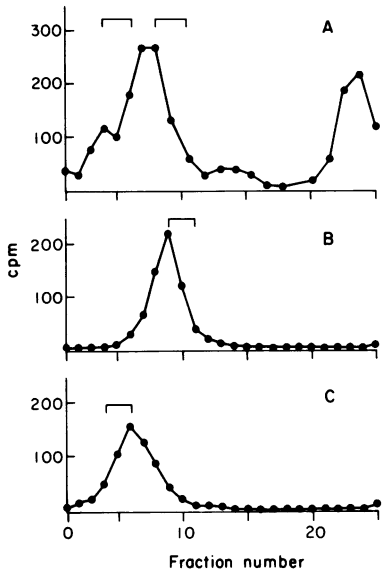

FIG. 5. *Fractionation procedures for virion RNA. (A) Total (^{3}H-labeled) RNA from purified RAV-2 virus particles was fractionated by velocity sedimentation (toward the left). RNA in the high-molecular-weight regions indicated by brackets was then refractionated. The most slowly (B) and most rapidly (C) sedimenting RNA in the resulting gradients (indicated by brackets) was then prepared in equal concentrations, denatured, and injected into 400 RSV(−) cells. The rapidly sedimenting RNA consistently promoted the release of from 10- to 20-fold more infectious virus than the slowly sedimenting RNA.*

otherwise identical RNA preparations. Association with the virion complex did not preclude the translation of microinjected RNA. This result might have been due to general dissociation of the RNA complex within the cytoplasm of infected cells, since helper virus constituted approximately 60% of the infectious virus released after injection of either denatured or undenatured 60–70S RNA. Studies by Cheung et al. (7) suggest that only 35S RNA is directly encapsulated into the virus particle. If so, the injected RNA complex was dissociated by the cell into 35S subunits, which were then encapsulated to form RAV-2 viruses.

DISCUSSION

RNA present in the avian leukosis virus particle expresses activity as viral envelope glycoprotein messenger. This observation is significant because the major 35S virion RNA species is totally inactive as *env* messenger. The evidence reported here suggests that the active RNA molecule within the virion is 21S *env* messenger (27) encapsulated by the virus particle directly from the infected cell. First, the virion molecule displayed physical and biological characteristics similar to the cellular species; the virion-associated messenger activity sedimented as a discrete species at 21S and promoted the same time course of infectious virus production after injection into RSV(−) cells as did *env* messenger from infected cells (unpublished data). Second, in these experiments translation was carried out by intact cells, and the product had to be biologically active to be observed. Only a natural messenger molecule would be expected to function properly in this system. Third, only 35S and 21S RNA species within infected cells contain *env*-specific sequences (14, 33). 21S *env* messenger could therefore appear within the virion either by direct encapsulation of 21S RNA or by cleavage of 35S RNA within the virus particle. Repeated attempts to generate active *env* messenger by in vitro fragmentation of 35S virion RNA by enzymatic and chemical means, however, have failed. Finally, rapidly sedimenting virion high-molecular-weight RNA was associated with proportionately more *env* messenger than slowly sedimenting virion RNA as further evidence against generation of the active virion messenger by fragmentation of 35S virion RNA.

The inactivity of fragments of 35S virion RNA after injection into RSV(−) cells is in contrast to the results reported with cell-free translation. Purchio et al. (22) found that 21S, polyadenylic acid-containing fragments of sarcoma virion RNA (generated by RNase T1) were active in a rabbit reticulocyte cell-free translation system and directed the synthesis of the 60K (presumptive *src*) protein. The fact that these fragments were translated well in the reticulocyte cell-free system, but similar fragments of RAV-2 virion RNA were not translated upon microinjection into living RSV(−) cells, suggests that RNAs that would not normally function within a living, homologous cell may be translated by a heterologous cell-free system.

It has been shown that RNAs other than viral 30–40S RNA can be encapsulated into retrovirus particles (5, 15). This work demonstrates that an mRNA molecule that contains viral-specific sequences is selectively encapsulated by the virus particle and becomes associated in a highly specific way with the virion high-molecular-weight RNA complex. The virion high-molecular-weight complex is composed of two 35S RNA subunits, viral RNA-dependent DNA polymerase (30) and the primer (6, 20, 24) for DNA synthesis (which is located near the 5′ terminus of the 35S subunit [11, 29]). Viral DNA synthesis initiates near the 5′ terminus of virion RNA,

proceeds to the 5′ terminus, and then continues, beginning at the 3′ terminus of the 35S RNA subunit (8, 13, 25). Ninety-five percent of the *env* messenger in RAV-2 virus particles is associated with this complex by a bond whose thermal stability is indistinguishable from that which stabilized the virion complex itself.

It is not known how the messenger-containing virion RNA complex might be arranged; whether the 21S molecule is trapped within the complex or displaces one of the subunits in some intermolecular bonds. Whatever the case, it is apparent that the 21S molecule is intimately associated with the virion RNA. Since *env* messenger from the cell contains both the 5′ and 3′ termini of virion 35S RNA, if it were encapsulated into the virus particle in close association with the high-molecular-weight complex, it might become involved in initiation and/or continuation of viral DNA synthesis, leading to the production of a subgenomic provirus. The resulting DNA might contain only nucleotide sequences of the *env* message; but since this would include the 5′ and 3′ termini of 35S virion RNA, it would presumably possess the information necessary for integration into host chromosomes and subsequent transcription into *env* messenger in the absence of other viral sequences.

Evidence that *env* messenger is encapsulated into the virus particle and becomes closely associated with the virion RNA complex might be helpful in understanding the long-term expression of *env* messenger after injection into RSV(−) cells. When 21S *env* messenger from RAV-2-infected cells was injected into RSV(−) cells, a peak in the rate of release of transforming virus was observed at 9 h, followed by a sharp decline, as expected for a phenomenon dependent upon a messenger with a relatively short half-life. The rate of infectious virus release did not decline to zero, however, but plateaued after approximately 2 days and began to increase for the next 36 h. At no time were helper virus produced by the culture, indicating that no known genetic manipulation could duplicate the phenomenon (27).

It is possible that some of the *env* messenger injected into RSV(−) cells became encapsulated into virus particles which then infected neighboring cells so as to produce a subgenomic, *env*-containing provirus as postulated above (which then became integrated and expressed by the cell). It may even be possible for a host messenger to acquire viral sequences sufficient to allow its participation in a similar phenomenon, resulting in gene duplication or constitutive expression of a random host function which could lead to cell transformation.

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