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An Association Between Globin Messenger RNA and 60S RNA Derived from Friend Leukemia Virus

(mouse/RNA tumor virus)

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ABSTRACT **The association between certain cellular RNAs and purified RNA tumor viruses prompted us to examine the possibility that specific host messenger RNAs might also be incorporated into RNA tumor viruses. Using a mouse cell line infected with Friend leukemia virus, T-3-Cl-2, which can be induced to accumulate mouse-globin messenger RNA, we show that mouse-globin messenger RNA sequences are present in viral particles purified from the culture medium of globin-producing cells. These globin messenger RNA sequences are absent from viral particles derived from T-3-Cl-2 cells that are not producing globin messenger RNA. Virus-associated globin messenger RNA sequences sediment in association with the 60S viral RNA complex as well as in free, 9S form. However, under mild denaturing conditions which result in the conversion of viral 60S RNA to 30S and smaller forms, all the globin sequences sediment as 9S RNA. Appropriate control experiments indicate that the virus-associated globin messenger RNA is resistant to degradation by exogenous ribonuclease; that exogenously added globin messenger RNA does not become associated with the 60S viral RNA complex; and that globin messenger RNA can be detected in virions derived from cells both induced for and constitutively synthesizing globin messenger RNA.**

A number of host proteins and nucleic acids have been found in association with RNA tumor viruses (see refs., 1–4). The role, if any, of these cellular components in viral replication or transformation is not known, but the detection of ribosomal and tRNAs associated with these viruses (5–15) prompted us to ask whether specific host mRNAs might be included in the viral particle as well. The fact that these viruses recombine or reassort genetic information in infected cells suggested this might be the case (16–19). Such an observation would be significant in several respects. It would provide further evidence for the inclusion of host-determined sequences in RNA tumor viruses, and would raise, at least formally, the possibility that these virions might serve as vectors of host-gene sequences during RNA virus infection. Further, the potential inclusion of host sequences in viral reverse-transcript DNA probes might influence conclusions drawn from studies designed to detect specific viral sequences in cellular RNA and DNA.

The cultured cell line infected with Friend leukemia virus (FLV), T-3-Cl-2, provides a convenient system in which to seek an association between a specific host mRNA and purified RNA virions (20). These cells, similar to those described by Friend (21), shed viral particles and can be induced to accumulate easily detectable amounts of intracellular mouse-globin mRNA when $(CH_3)_2SO$ is added to their culture medium (22, 23, and Ross *et al.*, manuscript in preparation). In the present studies we have used radioactive, synthetic DNA complementary to mouse-globin mRNA (globin [^{3}H]cDNA) (24–26) to demonstrate an association between mouse-globin mRNA and the complex RNA derived from purified viral particles.

Abbreviations: FLV, Friend leukemia virus; cDNA, complementary DNA.

MATERIALS AND METHODS

Purification of Viral Particles. T-3-Cl-2 cells were grown in the presence or absence of 1% (v/v) $(CH_3)_2SO$ for 4–5 days, as described (22). Growth was logarithmic through at least 3 days of treatment. Cl 745 cells (27), which are infected with FLV and make globin and globin mRNA in the absence of $(CH_3)_2SO$ (S. Orkin, personal communication), were grown under identical conditions, but without $(CH_3)_2SO$. Cell-free medium was collected, clarified, and stored at −80°C until sufficient material had accumulated for further processing. At that time, a second low-speed clarification was performed, and then viral particles were purified by two successive isopycnic sucrose gradients (15–60%). Viruses purified from treated and untreated cultures banded at a characteristic density of 1.15–1.16 g/cm^3.

Extraction of Total Viral RNA. Viral pellets were thawed at room temperature and suspended in a 0.1 volume of 10% (v/v) sodium dodecylsulfate. The viral suspension was extracted at room temperature three times with one equal volume each of phenol and of chloroform–isoamyl alcohol (24:1, v/v). The aqueous phase was then extracted twice more with chloroform–isoamyl alcohol alone. For all experiments, except those described in Fig. 2 (in which no carrier was added), 100 μg of *Escherichia coli* tRNA and three volumes of ethanol were added. After precipitation for 12–18 hr at −20° C, the RNA was centrifuged out of ethanol and resuspended in buffer A (0.1 M sodium chloride–10 mM Tris·HCl, pH 7.4–1 mM EDTA). RNA concentration was measured as absorbance at 260 nm, assuming 20 A_{260} units/mg of RNA.

Preparation of Complementary DNAs and Hybridization Assays. [^{3}H]DNA complementary to FLV RNA (FLV [^{3}H]-cDNA) was synthesized and purified as described (28), except that the DNA was labeled with [^{3}H]dCMP (Schwarz/Mann, 25.2 Ci/mmol). [^{3}H]DNA complementary to purified 9S mouse-globin mRNA (globin [^{3}H]cDNA) was synthesized and purified as described (25), with [^{3}H]dCTP of the same specific

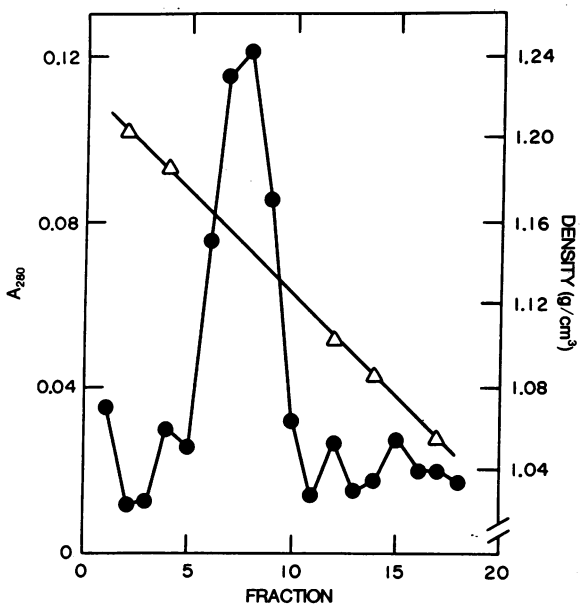

FIG. 1. Equilibrium density gradient centrifugation of partially purified FLV. Viruses that had been once banded by equilibrium centrifugation in a sucrose gradient were applied to a second linear 15–60% (w/v) sucrose gradient made in 0.1 M NaCl–10 mM Tris·HCl, pH 7.4–1 mM EDTA. Centrifugation was for 12 hr at 35,000 rpm in a Spinco SW41 rotor. Fractions were collected by drops from the bottom of the tube. Density was calculated from the refractive index. (△) Density (g/cm³); (●) A_{260}.

activity. Hybridization assays measuring resistance of hybrid molecules to degradation to nuclease S_1 were performed as described (22), with the following exception: some hybridization assays (see figure legends) were performed at 45°C in reaction volumes of either 0.03 or 0.06 ml containing 33% (v/v) formamide and 0.45 M NaCl.

Sucrose Gradient Centrifugation Analyses. For nondenaturing gradients, RNA samples precipitated with ethanol were dissolved in 0.25 ml of buffer A at 4°C. The RNA solution was then layered over a 12.1-ml linear sucrose gradient (15–30%, w/v) made in buffer A and centrifuged at 40,000 rpm for 3.5 hr in a Spinco SW41 rotor at 4°C. Approximately 0.6-ml fractions were collected by drops from the bottom of the tube. To each tube was added 50 μg of carrier tRNA and three volumes of ethanol. After 20 hr at −20°C, the RNA was centrifuged and dissolved in a small volume of water and used directly for hybridization analysis. For denaturing gradients, RNA was dissolved in 0.2 ml of buffer B (10 mM Tris·HCl, pH 7.4–10 mM EDTA), heated to 70°C for 5 min, and rapidly cooled in an ice-water bath. The RNA was then immediately applied to a 12.1-ml linear 15–30% (w/v) sucrose gradient made in buffer B and centrifuged at 40,000 rpm in an SW41 rotor at 4°C for 11 hr. Fractions were collected and precipitated as described above, except that each fraction was made to 0.15 M NaCl before precipitation. Fractions were then treated exactly as above.

RESULTS

The virus particles used for these studies were purified by two equilibrium density centrifugations in sucrose gradients. An example of the sedimentation properties of virus isolated by a single equilibrium sedimentation is shown in Fig. 1. The virus band is located at a density of approximately 1.158 g/cm³. No difference was observed in the banding patterns of virus isolated from induced [$(CH_3)_2SO$-treated] and nontreated T-3-Cl-2 cells (data not shown).

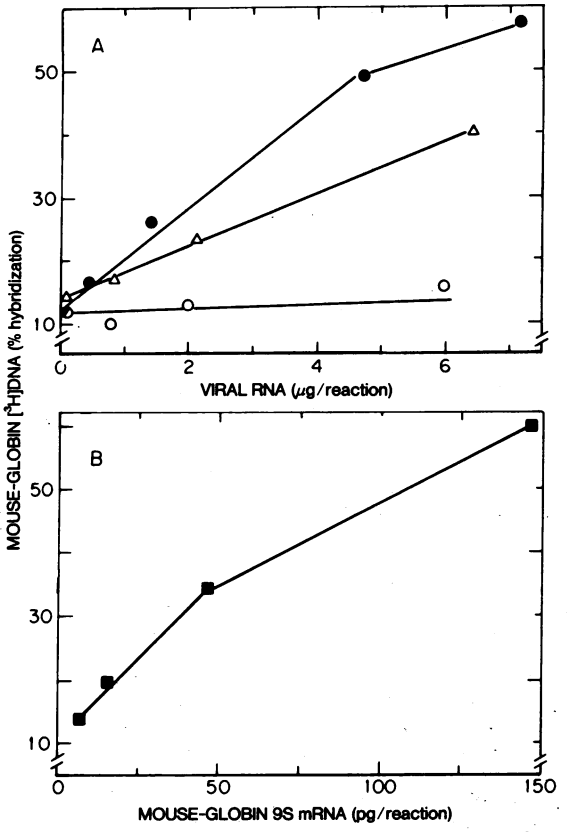

FIG. 2. Detection of globin sequences associated with FLV RNA by hybridization with globin [³H]cDNA. Total viral RNA was extracted from purified virions as described in *Methods*. No carrier RNA was added during the extraction procedure. Incubation was for 90 hr in 0.03 ml at 45°C in buffer containing 0.45 M NaCl and 33% (v/v) formamide. Percent hybridization was determined by the S1 nuclease assay. (*A*) Incubation with FLV RNAs. (○) FLV from T-3-Cl-2 cells, no $(CH_3)_2SO$ (noninduced); (●) FLV from T-3-Cl-2 cells, 1% $(CH_3)_2SO$(induced); (△) FLV from Cl 745 cells, no $(CH_3)_2SO$ (constitutive). (*B*) Incubation with purified 9S mouse-globin mRNA standard. RNA was purified from mouse reticulocyte polysomes by affinity chromatography and sucrose gradient centrifugation, as described (29).

In order to determine whether globin mRNA was associated with the purified viral particles, RNA was prepared from virions isolated from induced [$(CH_3)_2SO$-treated] and uninduced cells. This RNA was tested for its ability to hybridize with mouse-globin [³H]cDNA. As shown in Fig. 2A, RNA prepared from virus released by induced cells—cells known to be synthesizing globin mRNA—contained hybridizable globin mRNA sequences, whereas RNA isolated from uninduced cells did not. To assure that the association observed between Friend leukemia virions (FLV) and globin mRNA was not a trival consequence of addition of $(CH_3)_2SO$ to the media of infected cells, virus and RNA were prepared from a line of FLV-infected erythroleukemia cells, clone 745 (27), which constitutively produces globin mRNA (and consequently globin) and does not require $(CH_3)_2SO$ induction (S. Orkin, personal communication). As shown in Fig. 2A, viral RNA derived from these globin-producing cells also contains globin mRNA sequences.

TABLE 1. *Estimation of globin mRNA content of Friend leukemia virions*

Source of virions	% globin mRNA in total viral RNA	No. of globin molecules per viral 60S molecule
T-3-Cl-2[$(CH_3)_2SO$-induced]	0.0018	0.0012
Cl 745	0.0010	0.0007

These estimates are calculated from the data in Fig. 2. To determine percent globin mRNA, the amount of purified 9S mouse-globin mRNA required to achieve 30% hybridization (H_{30}) was divided by the amount of viral RNA that gave 30% hybridization. H_{30} values are as follows: 9S globin mRNA = 3.9×10^{-5} μg; T-3-Cl-2 = 2.2 μg; Cl 745 = 3.9 μg. The number of globin molecules per 60S viral RNA molecule was calculated as follows: Assuming a molecular weight of 10^7 for the 60S FLV RNA, the mass of one molecule of 60S RNA = 1.7×10^{-5} pg. If 60S RNA = 75% of total viral RNA (see Fig. 3), then there would be one molecule of 60S RNA per 2.3×10^{-5} pg of total viral RNA. 2.3×10^{-5} pg of total viral RNA from T-3-Cl-2 cells would contain 4.1×10^{-10} pg of globin mRNA ($2.3 \times 10^{-5} \times 1.8 \times 10^{-5}$). Assuming a molecular weight of 2×10^5 for globin mRNA, the mass of one globin mRNA molecule is 3.3×10^{-7} pg. Therefore, the number of globin mRNA molecules in an amount of total viral RNA containing one 60S molecule would be $[(4.1 \times 10^{-10})/(3.3 \times 10^7)] = 1.2 \times 10^{-3}$. Similar calculations were used to determine the number of globin mRNA molecules per 60S RNA of Cl 745 virions.

The globin mRNA associated with the viral RNA can be roughly quantitated by a comparison of its hybridization properties to that of purified mouse-globin mRNA (Fig. 2B and Table 1). Globin mRNA sequences account for a rather small proportion of the total FLV RNA, approximately $1\text{–}2 \times 10^{-3}$%, an amount equivalent to about one globin mRNA molecule per thousand 60S viral RNA molecules.

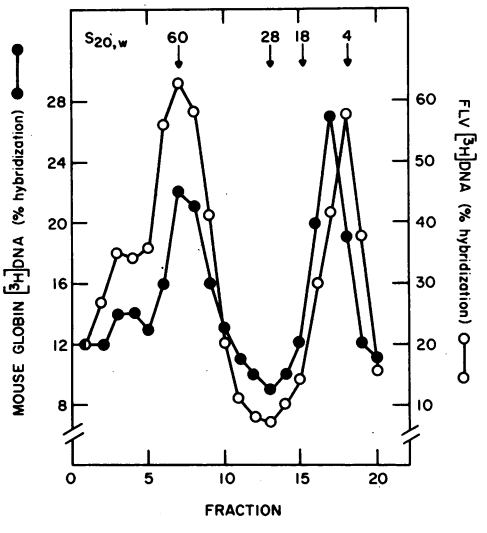

FIG. 3. Sedimentation analysis of globin mRNA associated with FLV. RNA was extracted from FL purified from T-3-Cl-2 cells grown in the presence of 1% $(CH_3)_2SO$. Centrifugation was for 3.5 hr in a linear 15–30% (w/v) sucrose gradient in buffer containing 0.1 M sodium chloride. Fractions were collected and concentrated to a final volume of 0.035 ml. For hybridization to FLV RNA, a 0.002-ml aliquot was incubated with 200 cpm (approximately 0.04 ng) of FLV [^{3}H]cDNA for 60 min in a 0.06-ml reaction volume. For hybridization to mouse-globin mRNA, a 0.03-ml aliquot was incubated with 375 cpm (approximately 0.05 ng) of globin [^{3}H]cDNA for 72 hr in a final volume of 0.06 ml. (○) FLV [^{3}H]cDNA; (●) globin [^{3}H]cDNA.

TABLE 2. *Insensitivity to ribonuclease of globin mRNA in FLV*

Source of virus	Additions	RNase	% Hybridization of [^{3}H]cDNA to RNA derived from virus mixtures: FLV [^{3}H] cDNA	Globin [^{3}H] cDNA
Treated cells	None	−	22	6
		+	25	5
Untreated cells	Mouse reticulocyte polysomes	−	36	75
		+	44	0
Untreated cells	9S Mouse-globin mRNA	−	40	77
		+	43	0

Approximately 5 A_{260} units of FLV purified from $(CH_3)_2SO$-treated T-3-Cl-2 cells were incubated in a 1.0-ml reaction mixture with or without 2 μg of RNase A for 45 min at 37°C. Controls contained FLV from untreated T-3-Cl-2 cells and either 5 μg of mouse reticulocyte polysomes (expt. 2) or 16 ng of purified 9S mouse globin mRNA (expt. 3). RNA was extracted and concentrated as described in *Methods*. Hybridization to mouse-globin [^{3}H]cDNA was in 0.15 ml for 96 hr, while hybridization to FLV cDNA was in 0.15 ml for 19 hr. Background (S1 nuclease-resistant cDNA alone) of 7% hybridization has been subtracted from the percent hybridization in the presence of RNA.

In an attempt to determine the nature of the association between globin mRNA and these viral particles, intact, purified virions containing globin mRNA were incubated together with bovine pancreatic ribonuclease (Table 2). The RNA prepared from these virions and that prepared from similarly treated, control virions from uninduced cells to which mouse reticulocyte polysomes or 9S globin mRNA had been added were assayed for ribonuclease-resistant globin mRNA and FLV RNA. In each case the amount of hybridizable FLV RNA was not reduced by ribonuclease treatment. Globin mRNA associated with isolated virions was also resistant to ribonuclease degradation, whereas exogenously added globin mRNA was completely sensitive to ribonuclease degradation. These results suggest that the globin mRNA is enclosed within the outer membrane of the virus.

This suggestion is further supported by the sedimentation properties of globin mRNA prepared from purified viral particles (Fig. 3). FLV RNA, as detected by hybridization to FLV [^{3}H]cDNA, is made up of the characteristic 60S and 4S components. Globin mRNA also sediments as two major components, one coincident with the heavy FLV RNA complex, the other with the 9S RNA fraction. The basis of this association between globin mRNA and the 60S FLV complex was further examined by denaturing the viral RNA complex under mild conditions that result in the conversion of the 60S viral complex into primarily 30S, intermediate, and 4S components (Fig. 4). After denaturation, the globin mRNA sedimented entirely with the 9S fraction, indicating that it was not covalently incorporated into a larger sequence.

The possibility that globin mRNA became associated with the 60S viral RNA complex in the course of RNA extraction

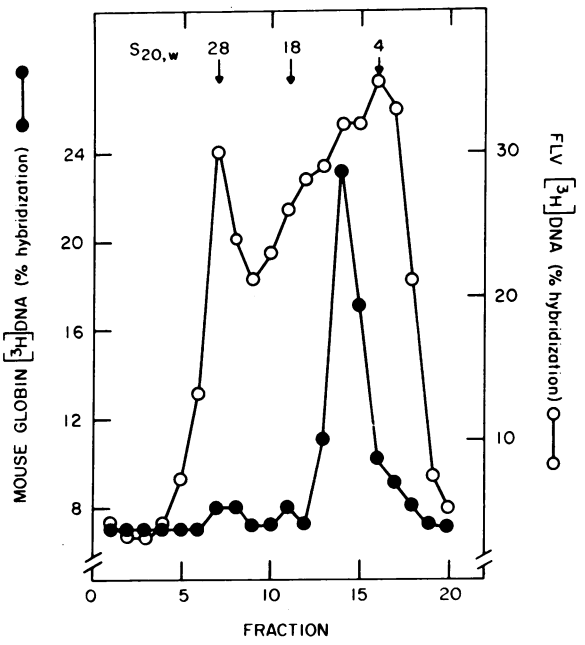

FIG. 4. Sedimentation analysis of globin mRNA associated with FLV after denaturation. RNA from FLV grown in $(CH_3)_2SO$-treated T-3-Cl-2 cells was heated and rapidly cooled in low-salt buffer. The heat-denatured RNA was then centrifuged for 11 hr at 4°C in a 15–30% sucrose gradient made in low-salt buffer. Fractions were collected and concentrated to 0.035 ml. A 0.002-ml aliquot was incubated with FLV [^{3}H]cDNA (210 cpm) for 1 hr, while a 0.03-ml aliquot was incubated with mouse-globin [^{3}H] CDNA (430 cpm) for 78 hr in 0.06-ml reaction volumes. (○) FLV [^{3}H]cDNA; (●) mouse-globin [^{3}H]cDNA.

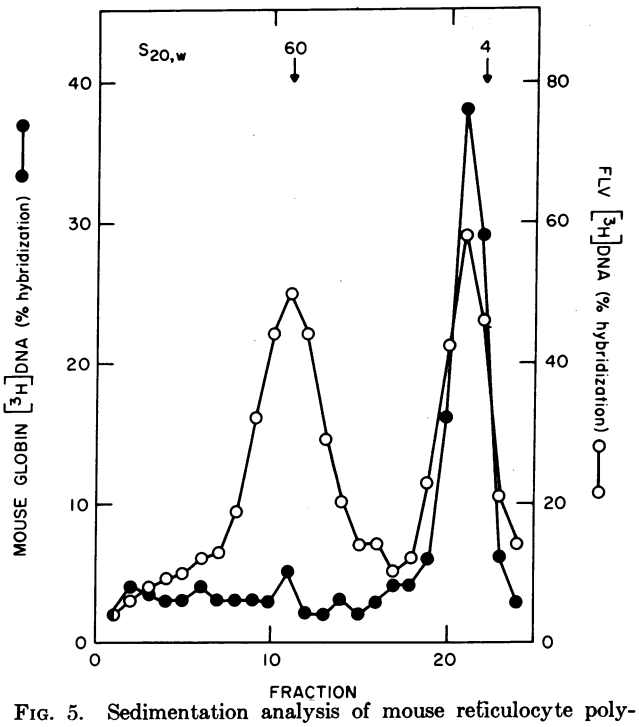

FIG. 5. Sedimentation analysis of mouse reticulocyte polysomal RNA added exogenously to FLV particles. Approximately 5 μg of mouse reticulocyte polysomes in 0.03 ml was added to an equivalent volume of FLV (containing approximately 0.3–0.5 A_{260} unit) that had been purified from T-3-Cl-2 cells grown without $(CH_3)_2SO$. The virus–polysome mixture was mixed gently and allowed to stand at room temperature for 10 min. RNA was then extracted as described in *Methods* and was centrifuged in a 15–30% sucrose gradient under conditions identical to those described in Fig. 3. Aliquots of 0.05 ml, taken directly from the gradient fractions, were incubated for 18 hr in a reaction volume of 0.15 ml with either FLV or globin [^{3}H]cDNA. (○) FLV [^{3}H]-cDNA; (●) mouse-globin [^{3}H]cDNA.

was examined by addition of reticulocyte polysomes to virions purified from the media of uninduced cells. RNA was extracted from this mixture and subjected to sucrose gradient centrifugation under nondenaturing conditions (Fig. 5; see Fig. 3). Under these conditions, the globin mRNA migrated entirely with the 9S RNA fraction.

DISCUSSION

Earlier studies of defective simian virus virions suggested that portions of the host chromosome could be covalently incorporated into simian virus 40 DNA (30). Further, hybridization analysis of this DNA indicated that it corresponded to the highly reiterated and unique (31) fractions of the mammalian genome. The RNA tumor viruses have been found to carry specific host ribosomal and tRNA sequences (5-15), but—in contrast to simian virus 40—these RNAs are not covalently incorporated into the viral genome. They occur both in association with the viral 60S RNA complex and in free configuration in the viral particle. Here we have provided initial evidence for an association between an mRNA corresponding to a specific structural mouse gene and the RNA complex of the Friend leukemia virus (Fig. 2). These globin mRNA sequences are resistant to exogenous ribonuclease (Table 2) and are associated with the 60S viral RNA complex (Fig. 3). In contrast, globin sequences added to virions as free mRNA or in reticulocyte polysomes, do not behave in this way (Table 2, Fig. 5). Taken together, these findings indicate that the association we observe is not a consequence of exogenous contamination of the purified virions.

Our hybridization studies indicate that one globin mRNA sequence is present per thousand viral genomes (see Table 1), a number roughly comparable to the number of unique *Escherichia coli* gene sequences present in the transducing bacteriophage P1 (32). Since globin sequences constitute 1.8×10^{-3}% of the total FLV RNA, it is not surprising that a reverse transcript made with FLV from induced T-3-Cl-2 cells fails to hybridize significantly to globin mRNA (J. Ross, unpublished observation). Nevertheless, it is possible that this mRNA is but one of many cellular mRNAs similarly incorporated into the virus. The mechanism of C-type virus formation might be expected to engulf a portion of the host cytoplasm (see reviews, 1–4). Further, the FLV particles used in these studies contain a *minimum* proportion of globin sequences since a portion of these virions were shed before the onset of globin mRNA accumulation, from cells induced for hemoglobin synthesis to various extents (22). Therefore, were each virion carrying a sample of host mRNA—as we argue it might—and were their sequences represented in the reverse transcript of the tumor virus RNA, hybridization with such a probe to detect *viral* sequences might view specific *host* sequences as well.

It seems unlikely that globin mRNA plays a specific role in the viral life cycle *per se*. Globin is not an obligatory constituent of T-3-Cl-2 cells nor—as we have shown—is its mRNA

found in viral particles derived from uninduced cells. The rather small proportion of virions that might carry globin mRNA strengthen this assumption. Nevertheless, such host RNAs—particularly mRNAs—might have some function as a class. For example, mRNAs containing reiterated host nucleotide sequences might provide nucleation sites for the linkage of viral RNA subunits during formation of the 60S viral RNA complex or, similarly, might serve as primers for RNA-directed DNA polymerase (33, 34). Perhaps these host components directly influence the fate of an infectious virion (35). Whatever their function, our observations raise at least the formal possibility that host mRNAs can be conveyed from one cell to another by the RNA tumor viruses. The presence of an enzyme in the virion capable of transcribing RNA into DNA (36) make this possibility an especially interesting one.

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