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Characterization of Polysome-Associated RNA from Influenza Virus-Infected Cells

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Virus-specific polysome-associated RNA (psRNA) and RNA after dissociation of polysomes were analyzed by direct hybridization with unlabeled viral RNA (vRNA) and complementary RNA (cRNA). psRNA after a 30-min pulse with [^{3}H]uridine contained 28% labeled (*) c*RNA, 70% host *RNA, and no v*RNA. After dissociation, ps*RNA sedimented heterogeneously. Heavy *RNA (>60S), ribosomal subunit RNA (r*suRNA, 30–60S), free mRNA (fm*RNA, 10–30S), and light *RNA (<10S) contained 16%, 54%, 70%, and 28% c*RNA, respectively, but no v*RNA. When actinomycin D (AcD) was added at 2 h postinfection, the nature of the psRNA depended on the concentration of AcD and the condition of the labeling. At AcD concentrations of 1 μg or more per ml, no detectable v*RNA or c*RNA was associated with polysomes. At 0.2 μg of AcD per ml (a concentration that partially inhibited cRNA synthesis) and 2 h of labeling at 2.5 h postinfection, ps*RNA contained 40% viral-specific RNA, which included both v*RNA and c*RNA in almost equal amounts. When polysomes were dissociated, however, viral-specific fm*RNA from AcD-treated cells contained exclusively c*RNA and no detectable v*RNA. Increasing amounts of labeled v*RNA were present in the heavy region of the gradient (and in the pellet), which also contained varying amounts of c*RNA. The labeled v*RNA appears to be associated with polysomes in a cesium chloride density gradient (ρ = 1.525 g/ml). Although we have ruled out the trivial explanation of viral ribonucleoprotein contamination, the nature of the complex containing both polysomes and v*RNA is unknown.

Several reports (4, 5, 7, 15, 17) indicate that complementary RNA (cRNA) may be the only functional messenger in influenza-infected cells, although some studies (10) suggest that viral RNA (vRNA) may be associated with polysomes along with cRNA. Some authors (20) also claim that vRNA can be translated in vitro. In none of these studies, however, was the intracellular vRNA assayed directly, possibly because of the lack of a suitable assay. Thus the question of whether, in addition to cRNA, a minority of vRNA segments was present in polysomes and/or served as messengers could not be ruled out. In this report, we have avoided these ambiguities by developing an assay for vRNA. We have assayed directly both vRNA and cRNA in polysome RNA (psRNA) and in free mRNA (fmRNA) isolated after dissociation of polysomes. We have analyzed RNA from infected cells with or without actinomycin D (AcD) treatment. Using these direct assays for both vRNA and cRNA, our results agree with the earlier finding (4, 5, 7, 15) that cRNA is the only functional messenger. Furthermore, we confirm the observation of Nayak (10) that vRNA, although not a functional messenger, is associated with polysomes.

MATERIALS AND METHODS

Virus and cells. WSN, a neurotropic variant of the WS strain of influenza A virus (HON1), and Kimber Farm cross K-137 embryonated chicken eggs were used throughout this study. The stock virus, prepared from 10- or 11-day-old embryonated chicken eggs, contained 4 × 10^8 PFU and 1,024 hemagglutinin units/ml (12).

Primary chicken embryo fibroblasts (CEF), prepared from 10- or 11-day-old embryonated eggs, were grown in prescription bottles with Eagle minimal essential medium (MEM) containing 10% tryptose phosphate broth (TPB) and 10% inactivated fetal calf serum (FCS). When confluent, the CEF monolayers were infected with WSN at an input multiplicity of 5 PFU/cell (45 min at 37°C) and covered with MEM medium containing 2% FCS and 5% TPB. In the labeling experiments, medium containing 2% dialyzed FCS was used.

To prepare radioactive virus, infected CEF monolayers (0.1 PFU/cell) were overlaid with MEM containing 2% dialyzed FCS and 10 μCi of [^{3}H]uridine

per ml. The cultured supernatant was harvested after 40 h of incubation at 37°C, freed from cell debris. Virus particles were concentrated by polyethyleneglycol precipitation (2) and further purified in sucrose gradients containing NTE buffer (pH 7.4) (12). vRNA was extracted by the sodium dodecyl sulfate-phenol method and analyzed in sucrose velocity gradients (12). Only the purified labeled vRNA from sucrose gradients was used for hybridization.

Isolation and analysis of RNA from infected cells. To isolate unlabeled cRNA, primary CEF were infected at 2 to 4 PFU/cell and treated with cycloheximide (10 μg/ml) at 2 h postinfection (p.i.) to preferentially inhibit vRNA synthesis (19). At 4 h p.i., RNA was extracted from the infected cells by the sodium dodecyl sulfate-cold phenol procedure (13). RNA was subsequently treated with DNase and reextracted and stored in ethyl alcohol (67%) at −20°C.

Hybridization procedure. The labeled RNA species were dissolved in 2× SSC buffer (0.3 M NaCl, 0.03 M sodium citrate). The position of the label in the RNA preparation is marked with an asterisk (e.g., v*RNA, c*RNA, etc.). Appropriate amounts of trichloroacetic acid-precipitable counts were used per 0.05 ml of total reactants. To assure saturating conditions, increasing concentrations (1×, 2×, 3×, etc.) of unlabeled RNA species (either total cell RNA containing cRNA prepared as above, or unlabeled vRNA extracted from purified virions) were added to a fixed amount of labeled RNA. Single-strength vRNA consists of about 0.2 μg of vRNA; 1× cRNA consists of 5 μg of cellular RNA. The reaction mixture (0.05 to 0.15 ml) was transferred into siliconized ampoules, sealed, and hybridized in a 78°C water bath for 1 h and then allowed to cool slowly to room temperature over a period of 18 h. The contents were removed from the ampoules, diluted with 1.0 ml of 2 × SSC buffer, and divided into two equal parts. RNase resistance was determined by treating one part with RNase A (20 μg/ml) and RNase T1 (20 units/ml) for 30 min at 37°C. All hybridization was done in duplicate and our data show the average values. There was no appreciable amounts of thermal degradation as determined by the loss of trichloroacetic acid-precipitable counts. The control samples were processed through the same hybridization procedure.

Isolation of polysome-associated labeled RNA. Cells were infected with 5 PFU/cell and treated with cycloheximide (2.5 μg/ml) for 5 to 30 min before harvest (21, 22). The monolayers were rinsed with cold TKM buffer (0.1 M KCl, 0.01 M Tris-hydrochloride [pH 7.5], 0.005 M $MgCl_2$) and kept thereafter either in ice or in the cold room. Cytoplasmic lysate was prepared by treating cells with deoxycholate (0.5%) and Tween 40 (1%) in TKM buffer (10) and analyzed in a 0.5 to 1.5 M linear sucrose gradient containing TKM buffer. Fractions of 0.8 ml were collected after scanning for optical density (OD) at 260 nm in a Gilford recording spectrophotometer. Aliquots were used for determining trichloroacetic acid-precipitable radioactivity.

Dissociation of polysomes into ribosomal subunits and mRNA. To obtain fmRNA, polysomes were isolated and subsequently dissociated into ribosomal subunits, releasing mRNA. EDTA dissociation did not provide a good separation of mRNA from rRNA because, unlike mammalian ribosomes, chicken ribosomes after EDTA treatment have a tendency partly to unfold and degrade into components sedimenting slower than 30S (F. O. Wettstein, unpublished data).

In vitro puromycin (PU) treatment, commonly used in dissociating polysomes in high salt-low Mg did not seem to be the best approach here, since low contamination of polysome preparation with RNase may lead to partial degradation of mRNA. We therefore established conditions under which a short exposure of PU had no effect on polysome isolation in TKM buffer, but these PU-treated polysomes dissociated completely in high salt-low Mg buffer, releasing ribosomal subunits (see Fig. 5). Accordingly, PU (4 μg/ml) was added for a short pulse (5 min) before harvest (3). Polysomes were isolated in TKM sucrose gradients, pooled, diluted 1:4 in TKM buffer, and pelleted through a 1.5 M sucrose cushion (SW50 rotor, 49,000 rpm for 4 h at 4°C). The pellet was suspended in a high salt-low Mg buffer (0.3 M KCl, 0.02 M Tris-hydrochloride [pH 7.5], 3×10^{-6} M $MgCl_2$), layered over a 0.5 to 1.5 M linear sucrose gradient in high salt-low Mg buffer, and centrifuged in an SW41 rotor at 27,000 rpm for 16 h at 4°C.

RESULTS

Characterization of a viral-specific cRNA probe. In previous studies (4, 5, 7, 15, 17) of influenza viral mRNA's, vRNA had not been assayed directly by hybridization using a cRNA probe; it had been measured only indirectly by self-annealing experiments. A conclusion of this type may be complicated by the presence of RNase-resistant cores of host RNA, of replicative intermediates, and of duplexes and by varying specific activity of the vRNA and cRNA pool, as well as by the possibly insufficient concentration of reactants required for the completion of hybridization.

To obtain a viral cRNA probe in vivo, we used cycloheximide at 10 μg/ml to inhibit vRNA synthesis (16, 19). Cycloheximide was added to infected cells 2 h p.i., and the total cell RNA was extracted 4 h p.i. Each batch of cRNA was preannealed and tested for viral-specific complementarity by hybridizing with [^{3}H]-uridine-labeled vRNA (v*RNA) extracted from purified virions. The results of two separate experiments are shown in Table 1. All of the v*RNA formed duplex when hybridized with preannealed cell RNA. It was concluded, therefore, that preannealed cell RNA prepared in the presence of cycloheximide contained free c*RNA.

However, in the above experiment it was not possible to quantify the amount of free cRNA,

TABLE 1. *Characterization of viral-specific cRNA from infected cells by hybridization to labeled vRNA (v*RNA)*[a]

RNA	Relative amt[b]	RNase/total (%Res)[c]	
		Expt 1	Expt 2
cRNA (preannealed)	1×	242/238 (101.2)	480/492 (97.6)
cRNA (preannealed)	2×	175/188 (93.0)	507/482 (105)
cRNA (preannealed)	4×	175/166 (105.4)	
v*RNA	0	5/250 (2.0)	11/495 (2.2)

[a] Total cell RNA was extracted at 4 h p.i. from infected cells treated with cycloheximide (10 μg/ml) at 2 h p.i. Isolated cellular RNA was preannealed. Equal amounts of purified v*RNA were added to the portions of preannealed cRNA and the mixture was hybridized as described in the text. All preparations including v*RNA control were treated exactly the same.

[b] Single strength (1×) is equal to 5 μg of cellular RNA.

[c] Counts per minute. Res, Resistance.

which was assayed next by hybridizing a constant amount of v*RNA to varying amounts of preannealed cell RNA. The amount of free cRNA was calculated from the slope and the plateau (Fig. 1). Approximately 0.115% of total cellular RNA was free cRNA (40 ng of cRNA/34.7 μg of cell RNA). The linearity of the slope indicated that hybridization indeed was measuring the amount of free cRNA. In another experiment, a large amount of uninfected cellular RNA (1 mg) was added in the hybridization mixture. Uninfected cellular RNA did not affect the hybridization of v*RNA to free cRNA.

In the next experiment, we added varying amounts of vRNA to a constant amount of cell RNA. Hybridization of labeled v*RNA decreased linearly as expected (Fig. 2), supporting the idea that in the preannealed cellular RNA, cRNA (and not vRNA) was the only virus-specific single-stranded RNA species available for hybridization. Finally, to determine whether vRNA (not c*RNA) hybridized exclusively to the preannealed cRNA preparation, RNA from infected cells (labeled at 3 to 5 h p.i.; 20 μCi of [^{3}H]uridine/ml) was isolated, denatured (5 min at 100°C), and hybridized to preannealed unlabeled cellular RNA in excess. After hybridization, labeled RNase-resistant RNA was isolated, denatured, and hybridized to unlabeled vRNA in excess. The results show that essentially all of the radioactivity in the duplex were displaced after denaturation and reannealing in presence of excess vRNA (Table 2). This experiment, therefore, confirmed that although labeled infected cell RNA contained both v*RNA and c*RNA, only v*RNA was forming duplex with unlabeled preannealed infected cellular RNA prepared in the presence of cycloheximide. The data in Table 2 also show that the efficiency of rehybridization was approximately 50%.

We therefore routinely used only preannealed cRNA in assaying v*RNA and the conditions of hybridization prevented any dissociation of preformed duplex. In some experiments, cRNA was further purified by polyuridylic acid-sepharose chromatography. Viral cRNA constituted about 2.5% total polyadenylic acid-containing cellular RNA. Any vRNA was further removed by self-annealing. Preannealed cRNA with or without polyuridylic acid-Sepharose chromatography produced identical results in assaying v*RNA.

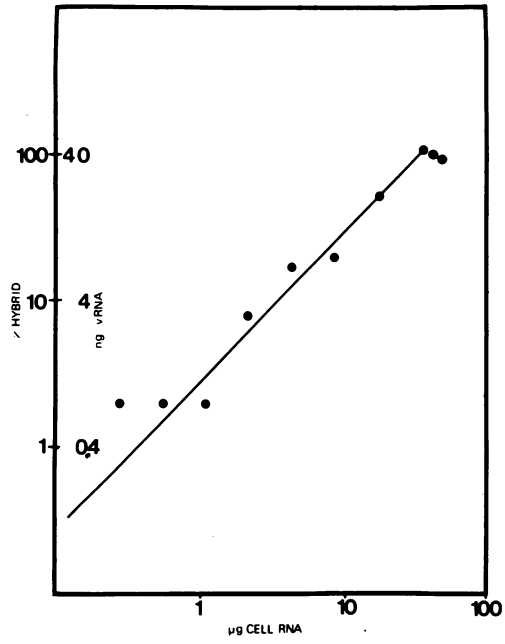

FIG. 1. *Quantitation of free cRNA in preannealed infected cellular RNA prepared in the presence of cycloheximide. Forty nanograms of labeled vRNA was hybridized to varying amounts of preannealed cellular RNA containing cRNA. The condition for hybridization is the same as in Table 1.*

Polysomes of infected cells. Initial studies indicated that there was no difference in the

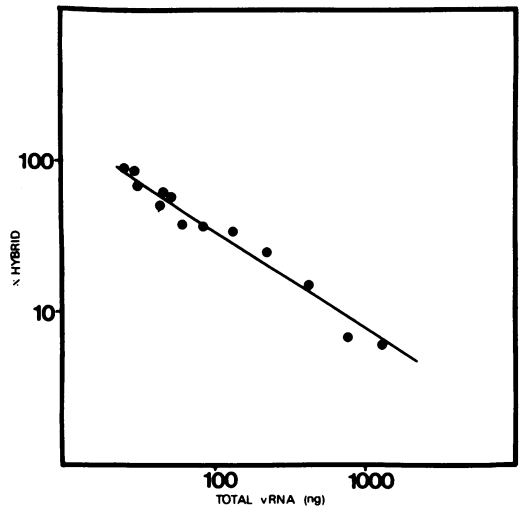

FIG. 2. *Specificity of hybridization between labeled vRNA and cRNA present in preannealed infected cellular RNA. Forty nanograms of vRNA (1,500 trichloroacetic acid-precipitable counts) was mixed with varying amounts of unlabeled vRNA and hybridized to a constant amount of preannealed cellular RNA containing cRNA (34 μg). The conditions for hybridization and determining hybrids are the same as in Table 1.*

TABLE 2. *Characterization of RNA hybridizing to preannealed cRNA preparation*[a]

Hybridization condition	RNase/Total (% Res)[b]
Not heat denatured	748/881 (84.9)
Heat denatured	41/873 (4.7)
Heat denatured	55/908 (6.1)
Heat denatured and self-annealed	371/668 (55.5)
Heat denatured and self-annealed	386/807 (47.8)
Heat denatured and annealed with 3 μg of vRNA	79/876 (9.0)
Heat denatured and annealed with 9 μg of vRNA	69/908 (7.6)

[a] Approximately 250 μg of preannealed unlabeled RNA from infected cells after cycloheximide treatment (described in Materials and Methods) was hybridized to the labeled cellular RNA obtained from one 100-mm infected dish (20 μCi/ml, 2 to 4 h p.i.). Approximately 20,000 cpm of RNase-resistant RNA was isolated after RNase treatment and phenol extraction. RNA was boiled 8 min in 0.01× SSC and cooled quickly. Aliquots of RNA (approximately 2,000 cpm) were distributed in ampoules and adjusted to a total volume (75 μl), salt (2× SSC), and vRNA concentration. Each aliquot was hybridized and analyzed for RNase resistance separately.

[b] Counts per minute. Res, Resistance.

optical density profile of uninfected and infected cells harvested 4 h p.i. (10). Preliminary experiments showed that treatment with cycloheximide at 2.5 μg/ml for up to 30 min did not affect the synthesis of vRNA or cRNA. To determine the effect of cycloheximide on the polysome profile, one set of infected CEF cells was treated with cycloheximide (2.5 μg/ml) for 30 min; the other set did not receive any drug treatment. Figure 3 shows the OD profile at 260 nm from both sets. Cycloheximide treatment caused an increase in the polysome yield (approximately 50%), particularly in fractions containing large polysomes. This is due to the fact that at concentrations lower than 10 μg/ml, cycloheximide does not totally abolish, but merely slows down, protein synthesis. Ribosomes attach to mRNA at normal rates, but chain elongation is inhibited, thus giving an apparent increase in the number of polysomes (21, 22). A short treatment with cycloheximide was therefore routinely used before harvesting polysomes.

Isolation and characterization of viral-specific RNA associated with polysomes. To analyze the nature of polysome-associated (ps*RNA), infected cells were labeled ([^{3}H]uridine, 20 μCi/ml) for 30 min in the presence of cycloheximide at 2.5 h p.i. The OD (at 260 nm) and the trichloroacetic acid-precipitable radioactivity profiles are shown in Fig. 4. For further analysis, fractions containing poly-

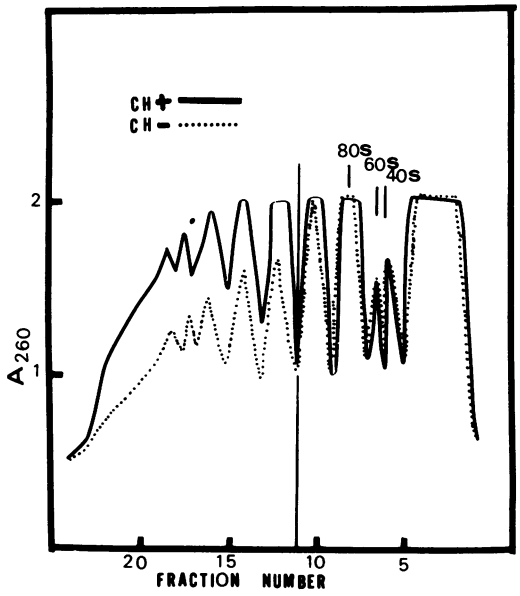

FIG. 3. *Effect of cycloheximide (CH) on the polysome yield of CEF. Cells in six 32-ounce (ca. 1-liter) bottles were infected. Three of them were treated with cycloheximide (2.5 μg/ml) for 30 min at 2.5 h p.i. Both sets were harvested at 3 h p.i.; cytoplasmic extract was isolated and analyzed simultaneously in parallel sucrose gradients (0.5 to 1.5 M) containing TKM buffers (SW27, 23,000 rpm for 10 h at 4°C). OD at 260 nm from both gradients was recorded. The vertical line marks the position of the dimer used for isolating polysomes in subsequent experiments.*

somes and ribonucleoprotein (RNP) (as shown by bar in Fig. 4) were pooled, and RNA was extracted (ps*RNA and RNP*RNA). The nature of ps*RNA and RNP*RNA was analyzed by hybridization.

Table 3 shows that ps*RNA contained approximately 30% of the label in cRNA and practically none in vRNA. The rest of the labeled RNA (70%) is likely to be of host origin, although it was not characterized. These results were in agreement with previous reports that the cRNA appears to be the only viral-specific RNA present in the polysomes. RNP*RNA contained 6% of the label in cRNA and 12% in vRNA (Table 3), and the rest was presumed to be the host RNA.

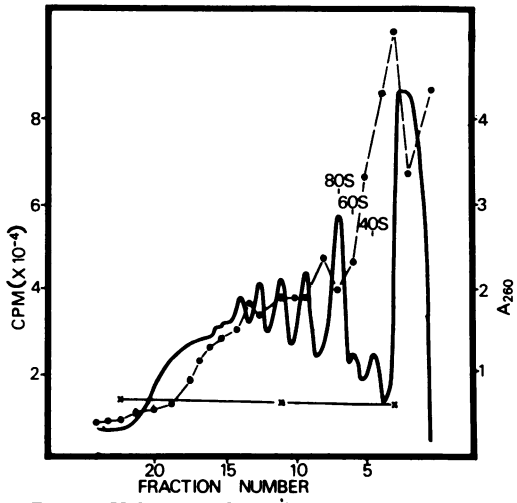

FIG. 4. *Velocity sedimentation analysis of [³H]-uridine-labeled cytoplasmic extract of infected cycloheximide-treated cells. Cells in three 32-ounce bottles were infected, labeled for 30 min with [³H]uridine (20 μCi/ml) at 2.5 h p.i. Cycloheximide (2.5 μg/ml) was also added at the same time (i.e., 30 min before harvest). The cell lysate was analyzed as in Fig. 3. The broken line represents trichloroacetic acid-precipitable radioactivity. The solid line represents the OD profile at 260 nm.*

Further characterization of ps*RNA was done in the following experiment. Infected CEF monolayers were labeled with [³H]uridine (20 μCi/ml) and pulse treated with cycloheximide (2.5 μg/ml) as in Table 3. In addition, PU (4 μg/ml) was added for 5 min before the 4-h harvest for reasons discussed in the Materials and Methods section. PU-treated polysomes were isolated from a TKM-sucrose gradient, dissociated, and analyzed in a high salt-low Mg sucrose gradient. The OD profile shows that in high salt-low Mg buffer, PU-treated polysomes dissociated completely, releasing ribosomal subunits (Fig. 5). The radioactivity profile shows the heterogeneous distribution of counts with a peak at the top of the gradients.

RNA was pooled and isolated from different fractions (as shown by bar in Fig. 5) and analyzed by hybridization. The results are presented in Table 4. Fractions containing fm*RNA (10–30S), ribosomal subunit RNA (r*suRNA) (30–60S), and heavy *RNA (>60S) contained approximately 70%, 50%, and 16% c*RNA, respectively, but no appreciable amount of v*RNA (<2%). These results agree with the recent finding of Pons (17) that much of influenza viral mRNA is not free but is associated with proteins. The RNA fraction at the top of the gradient (<10S) contained about 30% c*RNA and may represent smaller RNA segments (8 to 12S) or some degraded viral messengers. The rest (approximately 70%) is not hybridizable to either vRNA or cRNA probes and therefore may represent host RNA (possibly tRNA). The relatively high amount of hybridization with cRNA (7.7%) observed in this experiment was not reproducible in three other experiments in which this value was consistently less than 2%. These data show that under the above condition of labeling, no vRNA was

TABLE 3. *Characterization of [³H]uridine-labeled polysomal RNA (ps*RNA) and RNP*RNA of infected cells*[a]

Treatment	Relative amt	RNase/total (% Res)[b]	
		ps*RNA	RNP*RNA
Heat denatured		0/319 (0.0)	17/3,506 (0.58)
Self-annealed		10/332 (3.0)	292/3,580 (8.2)
Annealed with vRNA	1×	120/349 (34.4)	235/3,568 (6.6)
Annealed with vRNA	2×	70/308 (22.7)	225/3,676 (6.1)
Annealed with cRNA	1×	5/330 (1.5)	503/4,104 (12.3)
Annealed with cRNA	2×	5/283 (1.8)	463/3,737 (12.2)

[a] Cells were infected and labeled with [³H]uridine (10 μCi/ml) for 30 min before the 4-h harvest. Cycloheximide (2.5 μg/ml) was added for the last 15 min. A cytoplasmic lysate was prepared and layered on a sucrose gradient as described (Fig. 4). ps*RNA (fraction 11–22) and RNP*RNA (fraction 1–10) were extracted, precipitated, and hybridized to unlabeled vRNA or cRNA. All cRNA preparations were preannealed. vRNA 1× is 0.2 μg of vRNA; cRNA 1× is 5 μg of cellular RNA as in Table 1.

[b] Counts per minute. Res, Resistance.

detectable either in ps*RNA or in the RNA fractions after dissociation of polysomes.

Isolation and characterization of ps*RNA of infected cells treated with AcD. AcD suppresses the synthesis of cRNA (19) and thus increases the vRNA-to-cRNA ratio in infected cells when added 2 h p.i. Experiments were done to see whether AcD enhances the possibility of finding vRNA in polysomes, and finally in fmRNA after dissociation of polysomes. When infected cells were treated with AcD at 1 μg/ml and label was added 30 min later, neither c*RNA nor v*RNA was detected in the polysomes (16). It was obvious that cRNA synthesis did not occur under these conditions. In subsequent experiments, AcD was added at a lower concentration (0.2 μg/ml), which permits partial synthesis of cRNA. Radioactivity ([³H]uridine, 20 μCi/ml) was added simultaneously or at intervals (10 to 30 min) after pretreatment with AcD. All cultures were labeled for 2 h and cycloheximide (2.5 μg/ml) was added 15 min before harvest. Cytoplasmic extract from each set was analyzed in sucrose gradients. ps*RNA and RNP*RNA were isolated and analyzed by hybridization.

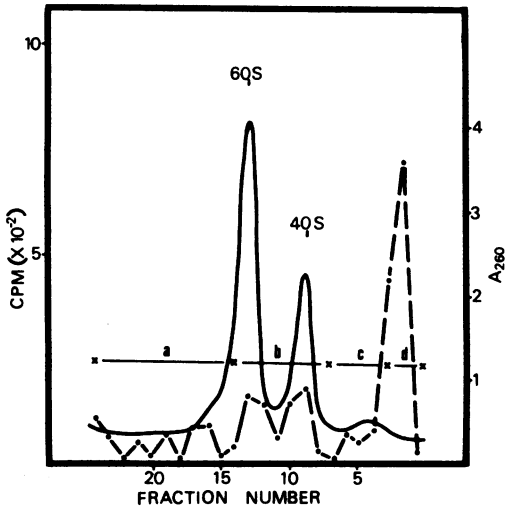

FIG. 5. *Velocity sedimentation analysis of [³H]-uridine-labeled polysomes after dissociation. Cells were infected and treated with [³H]uridine (20 μCi/ml) at 3 h, 30 min; cycloheximide (2.5 μg/ml) at 3 h, 45 min; PU (4 μg/ml) at 3 h, 55 min. Cells were harvested at 4 h p.i. Lysate was layered on a sucrose gradient as described; OD and radioactivity profiles of this gradient were comparable to those in Fig. 4. Fractions corresponding to the polysome region (as shown by bar in Fig. 4) were pooled, pelleted, resuspended, and analyzed in a high salt-low Mg⁺ gradient: the broken line (trichloroacetic acid-precipitable radioactivity) and the solid line (the OD profile at 260 nm). The bar indicates how the fractions were pooled for isolation and further analysis of RNA (Table 4). (a) Heavy *RNA (>60S); (b) rRNA (r*suRNA, 30-60S), (c) fmRNA (fm*RNA, 10-30S); (d) light RNA (<10S).*

The results from five different sets were essentially the same. A polysome profile is shown in Fig. 6A. As expected, less radioactivity and OD were present in the polysome region. The ratio of radioactivity in polysomes to RNP was also reduced in AcD-treated cells. The hybridization data are shown in Table 5. After 2 h of labeling, virus-specific ps*RNA in cells without AcD was predominantly cRNA although there was a small increase of radioactivity in v*RNA (1.7%, Table 3, versus 3.2%, Table 5). Furthermore, ps*RNA contained less radioactive c*RNA (34%, Table 3, versus 12%, Table 5) and presumably more host RNA, because the longer pulse permitted more radioactive host RNA to be processed and associated with polysomes.

In AcD-treated cells, ps*RNA contained 25 to 40% viral-specific RNA (i.e., both c*RNA and v*RNA) and the ratio of c*RNA to v*RNA was close to 1 (Table 5) whether the radioactivity

TABLE 4. *Characterization of [³H]Uridine-labeled RNA after dissociation of polysomes of infected cells*[a]

Treatment	Relative amt	RNase/total (% Res)[b]			
		fm*RNA	r*suRNA	Heavy *RNA	Light *RNA
Heat denatured		0/210 (0.0)	1/332 (0.3)	0/364 (0.0)	0/231 (0.0)
Self-annealed		0/225 (0.0)	9/340 (2.6)	8/378 (2.1)	0/238 (0.0)
Annealed with vRNA	1×	216/296 (73.0)	176/342 (51.5)	54/330 (16.4)	59/259 (22.8)
Annealed with vRNA	2×	195/300 (65.0)	160/294 (54.4)	54/364 (14.8)	66/234 (28.2)
Annealed with cRNA	1×	0/290 (0.0)	8/379 (2.1)	6/325 (1.8)	0/259 (0.0)
Annealed with cRNA	2×	0/237 (0.0)	6/316 (1.9)	4/312 (1.3)	19/245 (7.8)[c]

[a] Characterization of [³H]uridine-labeled RNA species of infected cells after polysome dissociation. Isolated polysomes were dissociated in high salt-low Mg gradient (Fig. 5). Four fractions (as shown by bars in Fig. 5) were separately pooled, extracted, and precipitated. Labeled fm*RNA, r*suRNA, heavy *RNA, and light *RNA were hybridized to varying amounts of unlabeled vRNA or cRNA. Hybridization conditions were the same as in Table 3.

[b] Counts per minute. Res, Resistance.

[c] In three other experiments, this value was less than 2%, indicating that high RNase resistance observed here is an experimental artifact.

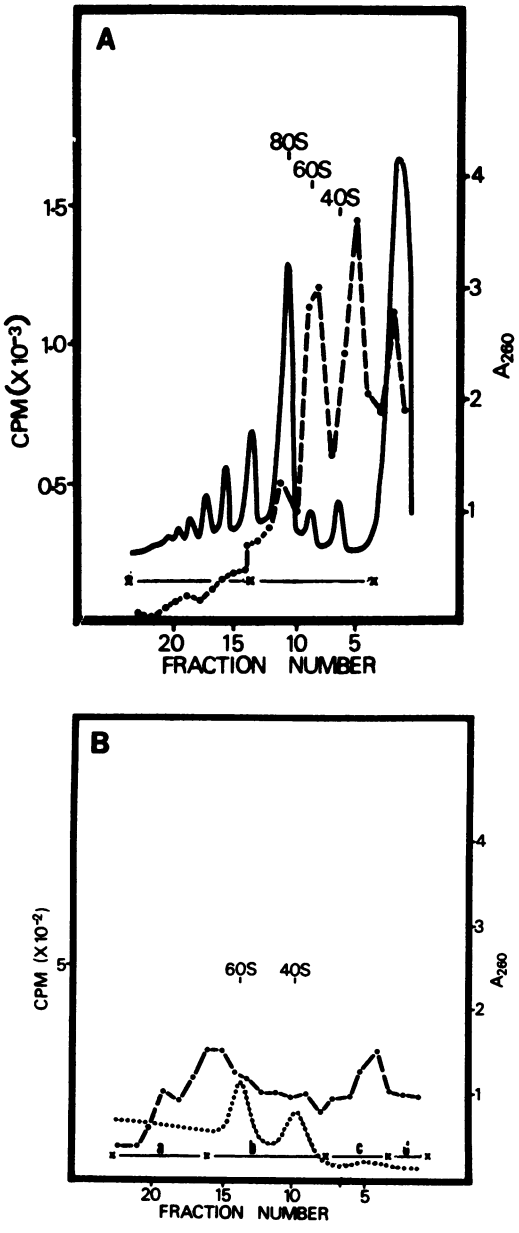

FIG. 6. *Velocity sedimentation of cytoplasmic extract of infected cells after AcD (0.2 μg/ml) treatment (A) and of dissociated polysomes (*ps) (B). (A) Five 32-ounce bottles of CEF were infected and treated with AcD (0.2 μg/ml) at 2 h, 10 min; [³H]uridine (20 μCi/ml) at 2 h, 30 min p.i.; cycloheximide (2.5 μg/ml) at 4 h, 15 min; and PU at 4 h, 25 min (4 μg/ml) were added. Cells were harvested at 4 h, 30 min (i.e., 2 h of labeling). Cell lysate was prepared and analyzed in a sucrose gradient (0.5 to 1.5 M) containing TKM buffer (SW27 large, 14 h, 30 min at 18,000 rpm). The OD profile at 260 nm (solid line) and trichloroacetic acid-precipitable counts (broken line) are plotted. (B) Fractions corresponding to the polysome region (A) were pooled, diluted 1:2 in TKM buffer, and pelleted in an SW51 rotor (50,000 rpm, 4 h). The polysome pellet was suspended in a high salt-low Mg buffer and layered on a sucrose gradient (0.5 to 1.5 M containing high salt-low Mg buffer) and centrifuged at 27,000 rpm, 14 h (SW41 rotor). The OD profile (dotted line) and the trichloroacetic acid-precipitable radioactivity profile (dash-dot line) are plotted. Fractions (as shown by bar) were pooled for isolating RNA.*

was added simultaneously or 30 min after AcD. These results confirmed previous observation by Nayak (10) that, under certain conditions at AcD treatment and labeling, both labeled vRNA and cRNA are found in polysomes.

RNP*RNA analyzed by hybridization showed a marked increase in virus-specific RNA (50 to 60%), although the amount of labeled c*RNA remained fairly constant (6%) (data not shown). The ratio of vRNA to cRNA in RNP*RNA became 12 or more, indicating that cRNA and host RNA synthesis relative to vRNA synthesis was drastically reduced by AcD treatment, even at 0.2 μg/ml (19). For further analysis, polysomes were isolated from PU-treated cells and analyzed in a high salt-low Mg sucrose gradient. Polysomes were comqletely dissociated with a heterogenous radioactivity profile (Fig. 6B). The majority of the counts in the heavy region again indicated a possible association of RNA with proteins, as described before. Fractions as shown by bars (Fig. 6B) were pooled, and RNA was isolated and assayed by hybridization with excess of vRNA and cRNA.

fm*RNA (10–30S) contained 40% c*RNA and less than 3%, if any, v*RNA, again showing that nearly all of the free viral messengers even in AcD-treated cells were cRNA (Table 5). The remaining 60% of label in the fmRNA is likely due to host mRNA, since 0.2 μg of AcD per ml may not inhibit all of host mRNA.

However, more v*RNA strands were present in heavier regions of the gradient, that is, about 5% in the r*suRNA fractions and about 6% in the heavy *RNA fractions, whereas the amounts of c*RNA present in r*suRNA and heavy *RNA were reduced to 29 and 15%, respectively (Table 5). In this experiment, although the amount of vRNA increased in the heavy region, its recovery was not complete after dissociation of polysomes. In a separate set of experiments, the pellet was found to contain the bulk of vRNA (51% labeled v*RNA and 25% c*RNA). The nature of this complex containing both v*RNA and c*RNA is unknown at present.

To determine if the labeled vRNA in the polysome area in AcD-treated cells was due to

TABLE 5. *Characterization of [^{3}H]uridine-labeled polysomes associated RNA from infected and AcD-treated cells*

Treatment	RNase/total (% Res)[a]						
	ps*RNA[b] without AcD	ps*RNA[b] with AcD	fm*RNA[c]	r*suRNA[c]	Heavy *RNA[c]	Light *RNA[c]	Pellet[d]
Heat denatured	21/2650 (0.8)	2/633 (0.3)	4/497 (0.8)	5/457 (1.1)	11/801 (1.4)	2/122 (1.6)	13/601 (2.2)
Self-annealed	81/2717 (3.0)	88/721 (12.2)	6/409 (1.5)	9/404 (2.2)	22/727 (3.0)	15/140 (10.7)	—
Annealed with vRNA (1×)	341/2634 (12.9)	133/677 (19.6)	159/423 (37.6)	84/391 (21.5)	103/686 (15.0)	90/156 (57.7)	125/586 (21.3)
Annealed with vRNA (2×)	389/3444 (11.3)	152/645 (23.6)	169/432 (39.1)	100/343 (29.1)	102/699 (14.6)	69/170 (40.6)	151/598 (25.2)
Annealed with cRNA (1×)	74/2912 (2.5)	128/622 (20.6)	11/375 (2.9)	13/366 (3.5)	39/609 (6.4)	5/162 (3.1)	285/523 (49.7)
Annealed with cRNA (2×)	84/2579 (3.2)	110/552 (19.9)	4/319 (1.2)	20/356 (5.6)	42/698 (6.0)	10/164 (6.1)	298/589 (50.5)

[a] Counts per minute. Res, Resistance.

[b] ps*RNA from cells with or without AcD were isolated from pooled fractions (as shown by bar in Fig. 6A) and hybridized with unlabeled vRNA and cRNA. RNP*RNA was also similarly analyzed, but the data (not shown here) are discussed in the text. Three other sets of cells with varying intervals of AcD pretreatment were also analyzed, and the results were essentially the same.

[c] Polysome fractions were pelleted and further dissociated in high salt-low Mg sucrose gradient (Fig. 6B). RNA was isolated from different regions (shown by bars in Fig. 6B) and analyzed by hybridization with both vRNA and preannealed cRNA.

[d] In a separate experiment, RNA was isolated from the pellet of a sucrose gradient containing dissociated polysomes and analyzed by hybridization.

contamination from viral RNP, ^{32}P-labeled virus was isolated, and RNP was separated and mixed with cytoplasmic extract. A number of experiments were done using either isolated RNP or disrupted viruses. The results were essentially the same. Viral RNP had an *S* value of 40–70*S* and sedimented in the region of single ribosome and ribosomal subunits, indicating that viral RNP could not be a major containment in the polysome area, because fractions smaller than dimers were not included in isolating ps*RNA. Furthermore, it was found that unlike the replicative complex of poliovirus RNA (6), polysome-associated influenza viral RNA with or without AcD treatment dissociated in low Mg^+ buffer (Nayak, unpublished data).

Finally, in another experiment, labeled polysomes were isolated, fixed with glutaraldehyde, and analyzed in a cesium chloride gradient (1). The radioactivity profile showed that the majority of counts had a density of polysomes (ρ = 1.525 g/ml) (Fig. 7). The RNA was isolated and analyzed in hybridization experiments. The results showed that, depending on the condition of the labeling in presence of AcD, 7 to 18% of label in ps*RNA was due to vRNA and had a density of polysomes (1.525 g/ml). These experiments further confirmed that vRNA, along with cRNA, was associated with polysomes.

DISCUSSION

Assay of viral RNA. In this report, we present a relatively simple procedure for assaying for vRNA. Although cRNA has been assayed directly by hybridization with labeled or unlabeled purified vRNA, a similar assay for measuring vRNA has not been used extensively. Scholtissek and Rott (18) had previously reported a procedure for assaying vRNA by using unlabeled cRNA in excess. They used microsomal fractions to isolate cRNA. In the method described here, cRNA is synthesized in infected cells in the presence of cycloheximide, which preferentially inhibits the synthesis of vRNA but not that of cRNA. Single-strand vRNA in the RNA preparation is further removed by preannealing into inactive duplex, leaving only the single-strand cRNA available for hybridization with vRNA. The data presented here show that hybridization of vRNA was specific and could be used for both quantitative and qualitative assay.

Nature of psRNA. Recent studies (4, 5, 15, 17) show that the majority of influenza viral messengers are cRNA. It has been shown that only cRNA contains polyadenylic acid (4, 11) methylated cap (R. M. Krug, personal commu-

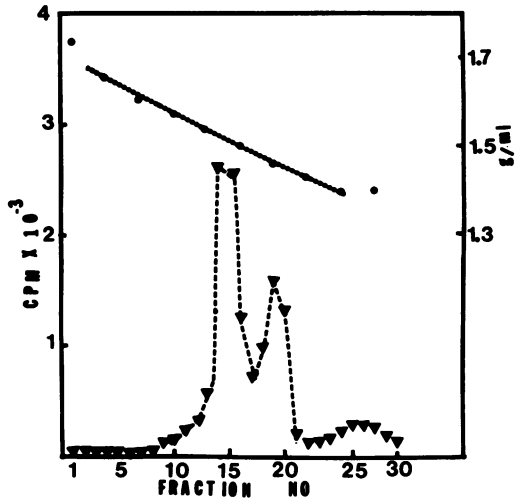

FIG. 7. *Analysis of polysomes in cesium chloride gradients. Polysomes were isolated from a sucrose gradient, fixed with glutaraldehyde, and centrifuged in a preformed cesium chloride gradient as described by Baltimore and Huang (1). RNA from fractions 14 and 15 was isolated (7) and analyzed by hybridization as shown in Table 6.*

TABLE 6. *Analysis of ps-RNA isolated from cesium chloride gradients*[a]

Treatment	RNase resistance (%)	
	Expt 1[b]	Expt 2[c]
Denatured	1.0	1.1
Annealed with cRNA[d]	7.4	15.0
Annealed with vRNA[e]	49.6	18.0

[a] RNA was isolated from CsCl gradients (ρ = 1.525 g/ml) (Fig. 7).

[b] In experiment 1, AcD (0.2 μg/ml) was added 10 min after the addition of [^{3}H]uridine (500 μCi/ml at 2.5 h p.i. for 2 h). A total of 2,100 trichloroacetic acid-precipitable cpm in duplicate were used per point.

[c] In experiment 2, AcD (0.2 μg/ml) was added simultaneously with [^{3}H]uridine; 900 cpm were used per point.

[d] Polyuridylic acid-selected, preannealed cRNA (5.0 μg) was used.

[e] Unlabeled vRNA (2.5 μg) was used.

nication) and can direct the synthesis of viral specific proteins in vitro (5; P. Palese, personal communication). Our data support these findings. By using direct hybridization assay for both vRNA and cRNA, we show that viral specific ps*RNA is entirely cRNA after a short pulse in absence of AcD.

The studies reported here confirm our earlier finding that when cRNA synthesis is partially inhibited, vRNA becomes detectable in the polysome region. However, after dissociation of polysomes, none of these vRNA is present in the form of fmRNA but is mostly associated with a larger complex. Recently, Etkind and Krug (5) have reported the presence of some vRNA as partial duplex in RNA isolated from polysomes and by oligo(dT)-cellulose chromatography. Glass et al. (7) have reported that some of the vRNA in the polysome regions could be due to contamination from viral RNP. However, our data show that a significant fraction of vRNA is clearly associated with polysomes both in sucrose gradients and in cesium chloride gradients. The nature of this association, however, is unknown at present. We are currently investigating if this polysome-associated vRNA is a part of a replication, transcription, or transcription-translation complex.

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