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Improved Method for Detecting Poliovirus Negative Strands Used To Demonstrate Specificity of Positive-Strand Encapsidation and the Ratio of Positive to Negative Strands in Infected Cells

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We have developed a ribonuclease protection method suitable for sensitive detection of an RNA species in the presence of a large excess of its complementary strand, as for the detection of negative strands of positive-strand RNA viruses. By using this method to probe for poliovirus negative strands in virions, we found that positive strands are present in at least 40,000-fold excess over negative strands. Thus, we have confirmed that poliovirus encapsidation is highly specific for positive strands and have demonstrated that the genome-linked protein VPg, which is covalently attached to the 5′ ends of both positive and negative strands, cannot be the sole determinant of RNA packaging. We tested the ratios of viral positive strands to negative strands in cells at different times during infection; this value ranged from approximately 40/1 to 70/1, being highest at 4 h and lower at 2 and 6 h postinfection.

The detection and quantitation of negative-strand RNA of poliovirus and other positive-strand RNA viruses is greatly complicated by the presence of the positive strands. The large molar excess of positive strands, which in poliovirus-infected cells outnumber negative strands by about 30- to 70-fold (1; this work), interferes with any hybridization method to detect negative strands. We have found that the detection of poliovirus negative strands by either dot-blot hybridization or standard RNase protection assays is neither sensitive nor even responsive to the amount of negative strands; in some cases we found that the signal actually increased with decreasing amounts of negative strands. We assume that the problems with sensitivity and quantitativeness also occur with Northern (RNA) blots, unless the positive and negative strands are completely separated on the gel. To overcome these problems, we have developed a two-cycle RNase protection procedure that removes the excess positive strands before probing is performed. Negative and positive strands are hybridized, treated with single-strand-specific ribonucleases, and then hybridized to radioactive probes and subjected to standard RNase protection. This two-cycle RNase protection method is responsive to negative strand concentration and is much more sensitive than standard RNase protection for detection of negative strands.

A comparison of the standard and the two-cycle RNase protection methods is shown in Fig. 1. The standard method (Fig. 1A) was modified from Sambrook et al. (10). Briefly, cytoplasmic RNA from poliovirus-infected or uninfected cells was denatured and hybridized with 75 fmol of a labeled RNA probe, designated 5601-RV, containing nucleotides 5601 to 5819 of poliovirus positive-sense sequence and 37 nucleotides of vector sequence. Excess probe was digested with RNases, and the products were displayed on a denaturing polyacrylamide gel. The signal clearly did not vary in the expected manner, i.e., with the amount of negative strand. More protection of the probe was seen with RNA from 10^6 cells than with greater amounts of RNA; in this and other experiments (not shown), the signal bore little relation to the amount of negative strand. We assume that the RNA probe did not reliably compete with the positive strands for hybridization to the negative strands, resulting in variable amounts of signal.

For two-cycle RNase protection, cytoplasmic RNA was hybridized in the absence of probe and then subjected to RNase treatment. After proteinase K treatment, phenol extraction, and ethanol precipitation, 75 fmol of 5601-RV probe was added, and the samples were put through the standard RNase protection procedure. In the first cycle, all negative strands should have hybridized to positive strands, leaving the excess positive strands to be digested with RNase. In the second cycle, equimolar amounts of positive and negative strands should have been present, making it possible for the radioactive probe to pair with the negative strands with minimal interference from the remaining positive strands. Figure 1B shows the two-cycle RNase protection method used on the same cytoplasmic RNA samples as in Fig. 1A; negative-strand RNA was readily detected by this method. Analysis of the gel by radioanalytic quantitation showed that the signal with this method was more than 100-fold higher than with standard RNase protection and that the signal was responsive to the amount of negative strand (Fig. 2). Quantitative information on relative negative-strand concentrations in different samples could be readily obtained by comparing the dilutions that gave the same amount of signal.

Others have probed for poliovirus negative strands with a similar method, but with no first hybridization step (1). We found that the signal was reduced by about 25% when the first hybridization step was eliminated from the two-cycle method (not shown); we do not know whether the signal still reflects RNA concentration with this procedure. Since the utility of this method depends on having the negative strands already paired for the initial RNase treatment, it may give variable results with different extraction procedures or with virus mutants that alter the extent of pairing of negative strands in the cell.

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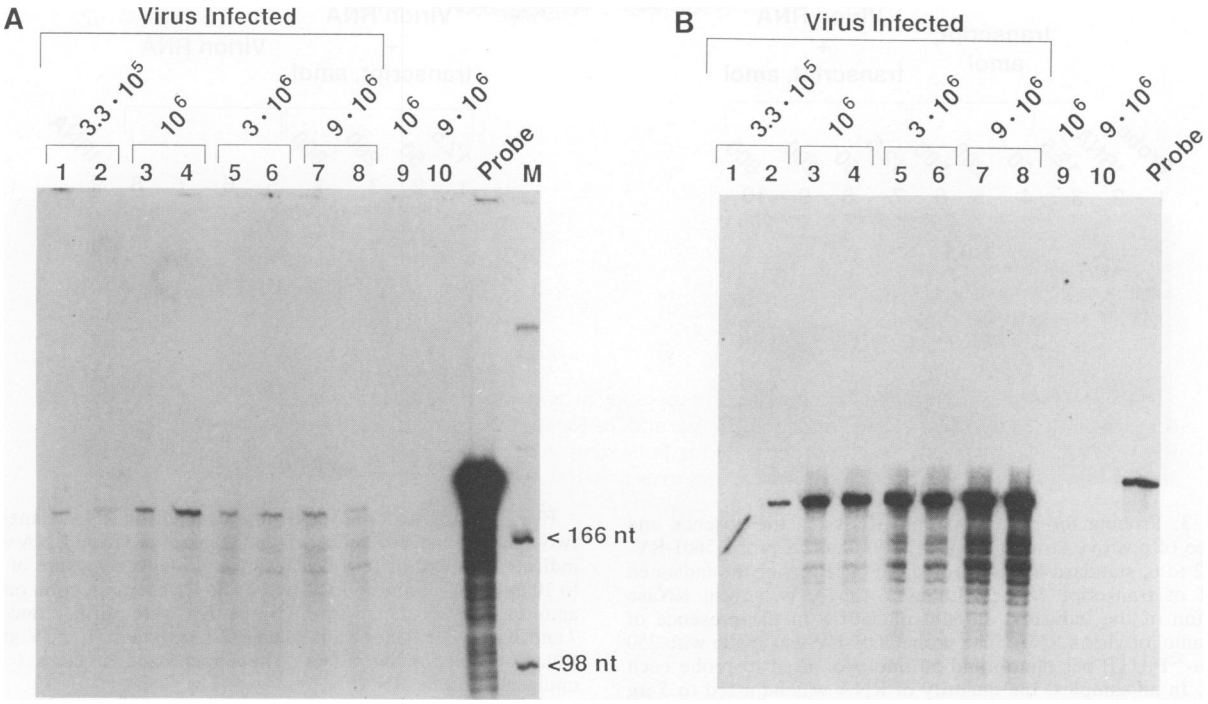

FIG. 1. Comparison of standard RNase protection and two-cycle RNase protection in probing for poliovirus negative strands. (A) Standard RNase protection performed on RNA from poliovirus-infected cells (lanes 1 to 8) and uninfected cells (lanes 9 and 10). The method was as described in Sambrook et al. (10), with the following modifications. Cytoplasmic RNA (prepared as previously described [5]) from the indicated number of poliovirus-infected cells 6 h postinfection or from uninfected cells was suspended in 30 μl of hybridization buffer containing 75 fmol of probe 5601-RV. The samples were denatured at 85°C for 5 min and hybridized overnight at 60°C. RNase digestion was at 7 to 8°C for 60 min in RNase mixture (500 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 4.5 μg of RNase A per ml, and 350 U of RNase T_1 per ml). The samples were treated with proteinase K, phenol extracted and ethanol precipitated with 0.25 μg of tRNA, and then loaded onto 10% polyacrylamide–7 M urea gels. Lane Probe, 0.25 fmol of probe 5601-RV; lane M, RNA markers. (B) Two-cycle RNase protection performed on RNA from poliovirus-infected cells (lanes 1 to 8) and uninfected cells (lanes 9 and 10). Cytoplasmic RNAs were hybridized overnight at 60°C in the absence of probe and then subjected to RNase treatment under the conditions described above. After proteinase K treatment, phenol extraction, and ethanol precipitation with 5 μg of tRNA, 75 fmol of probe in hybridization buffer was added, and the samples were put through the standard RNase protection procedure. Lane Probe, 0.25 fmol of probe 5601-RV; lane M, RNA markers. The autoradiogram in panel A was exposed 30 times longer than that in panel B. The probe 5601-RV was prepared by gel purifying an SP6 transcript of *Eco*RV-cut plasmid pSP6 5601-7510 made with 500 Ci of [α-^{32}P]UTP per mmol. The plasmid (a gift from Scott Diamond) was constructed by inserting nucleotides 5601 to 7510 of the poliovirus cDNA (8) into pGEM-3 (Promega).

To characterize the two-cycle RNase protection assay further and to test whether detection of negative strands with the two-cycle method is as efficient as probing done in the absence of positive strands, we performed a reconstruction experiment. An unlabeled RNA transcript of negative sense, 5601-S, representing poliovirus nucleotides 5601 to 7440, was prepared by in vitro transcription. Various amounts of this transcript were probed by the standard RNase protection assay (Fig. 3, lanes 3 to 6), using 50 fmol of labeled probe 5601-RV. In lanes 7 to 10, the negative-sense transcript was mixed with 80 fmol (0.2 μg) of virion RNA and subjected to the two-cycle RNase protection method.

A comparison of the amount of probe protected in these two methods (Table 1) showed that the two-cycle method yielded the amount of protection expected from probing done in the absence of positive strands, at least for 200 amol (2×10^{-16} mol) or less of transcript. Thus, for these samples, there was no significant loss of negative strands in the first cycle of the two-cycle procedure, and the positive strands remaining in the second cycle did not interfere significantly with negative-strand detection. In contrast, with 800 amol of transcript the two-cycle method gave significantly less protection than expected, probably because

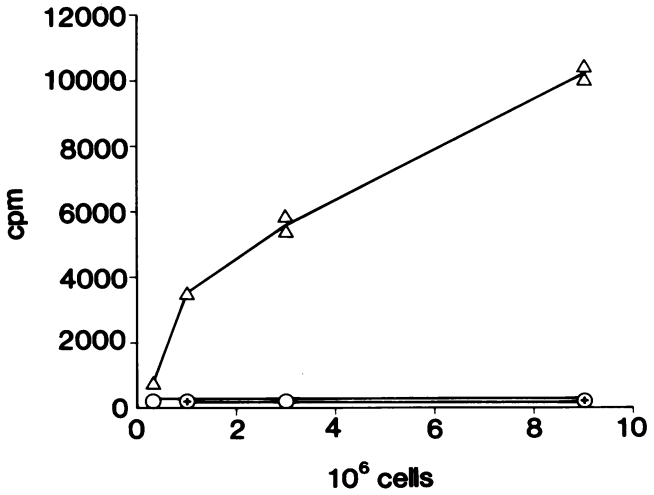

FIG. 2. Amount of probe protected in RNase protection assays. The radioactivity in the bands shown in Fig. 1 was determined by using the Ambis β particle detection system (Ambis System Inc., San Diego, Calif.). Triangles, two-cycle method; circles, standard method on RNA from infected cells; crosses, standard method on RNA from uninfected cells.

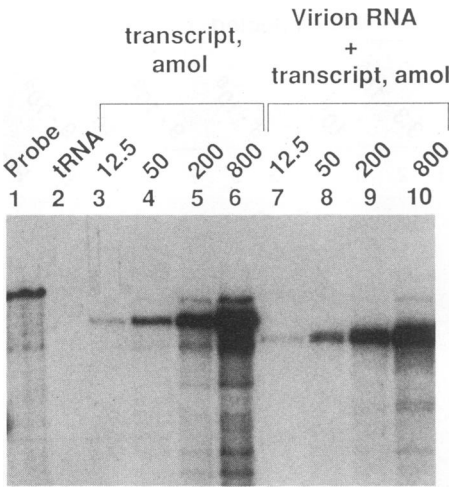

FIG. 3. Probing for negative-strand RNAs in the absence and presence of positive strands. Lane 1, 250 amol of probe 5601-RV. Lanes 2 to 6, standard RNase protection of tRNA or the indicated amount of transcript 5601-S. Lanes 7 to 10, two-cycle RNase protection of the indicated amount of 5601-S in the presence of 80,000 amol of virion RNA. The probe 5601-RV was made with 250 Ci of [α-^{32}P]UTP per mmol, and 50 fmol was used to probe each sample. In all samples, the quantity of RNA was adjusted to 5 μg with tRNA. Virion RNA was prepared as previously described (2) from virions that were purified twice on CsCl gradients. Virion RNA concentration was determined by spectrophotometry. 5601-S RNA was prepared by T7 transcription of *Sal*I-cut pSP6 5601-7510. The RNA concentration was measured from the amount of RNA synthesized in a parallel reaction containing a small amount of ^{32}P-labeled UTP and by comparison of ethidium bromide staining on a gel with RNA markers of known concentration. Because 5601-S is complementary to the probe in 12 nucleotides of polylinker sequence as well as in 206 nucleotides of poliovirus sequence, a 218-nucleotide fragment of probe was protected by standard RNase protection. In the two-cycle method, only the poliovirus-specific sequences of 5601-S were protected from RNase by virion RNA in the first cycle, so only the 206 nucleotides of the probe's poliovirus-specific sequences were protected.

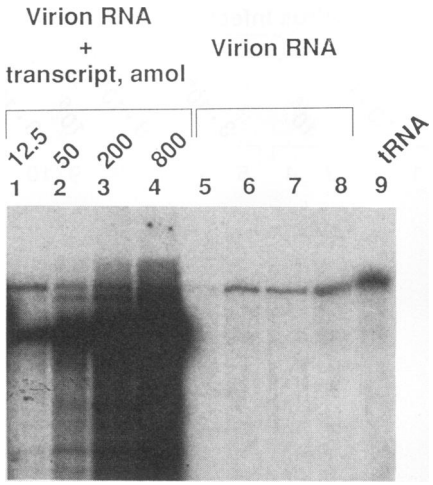

FIG. 4. Probing for negative strands in virion RNA. Lanes 1 to 4, two-cycle RNase protection on 80,000 amol of virion RNA with the indicated amount of 5601-S transcript (a longer exposure of lanes 7 to 10 in Fig. 3). Lanes 5 to 8, two-cycle RNase protection on 80,000 amol of RNA extracted from virions that were purified once (lanes 7 and 8) or twice (lanes 5 and 6) on a CsCl gradient. Lane 9, standard RNase protection on tRNA. The upper band in lanes 1 to 9 is undigested probe RNA.

the positive strands remaining after the first cycle (presumably 800 amol) interfered with the hybridization of the 50 fmol of probe to the negative strands. In competing with probe for hybridization to negative strands, full-length positive strands have a kinetic advantage over the shorter probe; the rate of hybridization varies with length, since longer RNAs have more sites with which to nucleate hybridization (4). Full-length RNA duplexes probably also have a thermodynamic advantage over multistranded structures containing the probe and full-length positive strands paired in regions outside the probe. These kinetic and thermodynamic advantages of positive strands must also contribute to the extremely poor detection of negative strands by conventional hybridization methods, even when they are performed with a vast molar excess of probe over negative strands.

TABLE 1. Efficiency of the two-cycle method

Amt of transcript used (amol)	Amt of probe protected (cpm)[a]		
	Standard method (no positive strands)	Two-cycle method (expected)[b]	Two-cycle method (actual)
12.5	10	8	11
50	39	30	32
200	160	123	120
800	584	450	312

[a] Counts per minute in bands of protected probe in Fig. 3 were determined by AMBIS radioanalytic quantitation, with counts from probing tRNA subtracted as background. In all cases, the standard deviation for the counts per minute was less than 15% of the amount stated.

[b] Calculated from counts protected in the standard method based on leaving behind 18% of the aqueous phase in the extra phenol extraction in the two-cycle method and on the protected fragment being 6% shorter.

We used the two-cycle RNase protection method to test rigorously whether poliovirus negative strands are packaged. Previous research, which tested the fraction of virion RNA that is resistant to single-strand-specific RNase after hybridization, indicated that no more than 8% of virion RNA could be negative strands (11). Since negative strands constitute less than 8% of total viral RNA in infected cells, this result says nothing about the specificity of encapsidation for positive strands. Tests on the infectivity of virion RNA after RNase treatment have failed to detect any double-stranded RNA that would be indicative of negative strands (3, 6), but it is possible that some negative strands were present but not hybridized to positive strands. RNase T_1 fingerprints of virion RNAs have likewise failed to show any evidence of negative strands (7), but T_1 fingerprinting is typically not very sensitive for detecting minor species. Thus, although it is generally believed that encapsidation is specific for positive strands, there is very little experimental justification for this belief. Furthermore, recent evidence consistent with the idea that VPg, a small protein covalently linked to the 5′ ends of both positive- and negative-strand poliovirus RNA, may be required for RNA packaging (9) suggests a mechanism by which negative strands may be packaged.

To test more precisely whether negative strands are packaged, 80 fmol of virion RNA was probed with 5601-RV. We failed to detect the 206-nucleotide band that negative strands would have protected (Fig. 4, lanes 5 to 8). To establish the

TABLE 2. Amount of positive- and negative-strand poliovirus RNA throughout infection

Time postinfection (h)	No. of cells probed	No. (molecules/cell) of[a]: Positive strands	No. (molecules/cell) of[a]: Negative strands	Ratio of positive to negative strands[b]
2	2.2×10^4	3,780		45
	6.7×10^4	3,780		
	2.0×10^5	3,360	78	
	6.0×10^5		120	
	1.8×10^6		84	
4	2.2×10^4	79,500	1,080	66
	6.7×10^4	72,300	1,100	
	2.0×10^5	31,700	640	
6	2.2×10^4	59,000	1,740	36
	6.7×10^4	62,800	1,730	
	2.0×10^5	31,400	1,030	

[a] The number of molecules of RNA was determined by comparing the counts of probe protected by cytoplasmic RNA with the counts in 0.5 fmol of the appropriate probe run on the same gel with the RNase protection samples. Most values shown are the average of two samples.

[b] To calculate this ratio, the values representing 6.7×10^4 cells were used (except for negative strands at 2 h, for which the value representing 1.8×10^6 cell sample was used). These values were chosen because they were in the linear range of the assay. This ratio for virions is >40,000 (see Fig. 4 and text).

limits of detection, this experiment was done in parallel with the reconstruction experiment described above, that of probing negative-sense transcripts mixed with excess virion RNA. Lanes 1 to 4 in Fig. 4, showing RNase protection on 12.5 to 800 amol of negative-sense RNA, show a longer exposure of lanes 7 to 10 in Fig. 3. We estimate that a band one-sixth the intensity of that in lane 1 would have been clearly visible, so 2 amol of negative strands would have been detectable in 80,000 amol of virion RNA. No negative strands were observed in lanes 5 to 8; therefore, positive strands must outnumber negative strands by at least 40,000-fold in this virion preparation.

Finally, we used RNase protection to assess the ratio of positive to negative strands at different times in the infectious cycle. Cytoplasmic RNA was prepared at 2, 4, and 6 h after poliovirus infection at a multiplicity of infection of 25. Standard RNase protection was used for positive strands, with 25 fmol of probe 158-H3 (containing nucleotides 7332 to 7440 of poliovirus negative strands, prepared with 500 Ci of [α-^{32}P]UTP per mmol). Two-cycle RNase protection was used for negative strands, with 75 fmol of probe 158-RI (the complement of 158-H3, prepared at the same specific activity). Various dilutions of cytoplasmic RNA were probed, and the number of RNA molecules was calculated by comparing the counts per minute of probe protected with the counts per minute in 0.5 fmol of probe that was run on the same gel. The results are shown in Table 2. For each time point, the counts protected by the more dilute samples were proportional to the amount of cytoplasmic RNA probed; we chose values from this linear range of the assays to calculate the ratio of positive to negative strands. We found the ratios to be 45, 66, and 36 at 2, 4, and 6 h postinfection, respectively. These values are slightly higher than the previously reported (1) ratio of 30 at 6 h postinfection at a multiplicity of infection of 1. Thus, the ratio of positive to negative strands does not vary dramatically after 2 h postinfection.

The increase in the ratio of positive to negative strands at 4 h may be due to packaging of positive strands, resulting in a reduction of the number of templates for negative-strand synthesis. The decreased ratio at 6 h could have several explanations, including the disappearance of a factor either needed for positive- but not negative-strand synthesis or needed in greater amounts to make positive strands.

Since negative strands make up at least 1.5% of viral RNA but are outnumbered by at least 40,000-fold in virions, encapsidation must have at least a 600-fold preference for positive strands over negative strands. Thus, VPg, present on RNA of both senses, cannot be the sole determinant of packaging. Instead, specific encapsidation of the positive strand must be a result of its sequence or structure, or it must be enabled by the presence of positive strands free of replication complexes.

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