Small RNAs of Rous Sarcoma Virus: Characterization by Two-Dimensional Polyacrylamide Gel Electrophoresis and Fingerprint Analysis

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Approximately 15 to 20 different species of small (4 to 7S) RNAs have been purified by two-dimensional polyacrylamide gel electrophoresis of RNA isolated from virions of Schmidt-Ruppin D strain of Rous sarcoma virus. Each species of small RNA has been isolated free of 70S RNA; nine of them, including 5S and 7S RNAs, were also found associated with the 70S genomic RNA. Most of the 4S RNAs are present at an average of less than one copy per virion. The 4S RNAs have T1 RNase (EC 2.7.7.26) fingerprints, which are very similar to those of tRNAs. One of the smallest 4S RNAs, which can act as a primer for initiation of RNA-directed DNA synthesis, is associated with the 70S RNA in 1 to 2 copies per complex, whereas an additional 6 to 8 copies of this molecule are free.

Virions of avian leukosis-sarcoma viruses contain approximately 30 to 100 molecules per virion of small (4 to 7S) RNAs, in addition to the high-molecular-weight genomic RNA (HMW-RNA) (3, 6, 11, 12, 15, 24). Denaturation of HMW-RNA followed by fractionation and sucrose gradient centrifugation reveals that some small RNAs are associated with HMW-RNA (14).

Relatively little is known about the virion low-molecular-weight RNAs and the roles they have in infection and transformation of cells. A variable fraction of this material probably arises from general degradation of virion HMW-RNA and is without function. Some discrete molecules, such as 7S, 5S, and transfer RNAs are quite likely cellular in origin (4, 16, 21, 23, 25); they may have specific functions, or they may be included in the virions upon budding, with no precise function. Virion transfer RNAs are capable of undergoing aminoacylation *in vitro* (9, 13, 26, 29-31). One of the small RNAs can serve as a primer for RNA-directed DNA synthesis (5, 8; A. J. Faras, J. M. Taylor, W. E. Levinson, H. M. Goodman, and J. M. Bishop, *J. Mol. Biol.*, in press).

Purification and characterization of individual species of small RNAs are required for a full understanding of the roles that they might play in infection and transformation of cells. Prepa-

ration of purified individual RNAs would provide information about the relative amounts of these RNAs and their location in the virion and the host cell and might permit assignment of particular functions to individual species. Because of difficulties in obtaining sufficient material for use with available separation techniques, previous work on small RNAs of avian RNA tumor viruses has been restricted primarily to studies on mixtures of molecules.

We have studied small RNAs of Schmidt-Ruppin-D strain of Rous sarcoma virus (SRD-RSV) by a two-dimensional polyacrylamide gel electrophoresis method which was developed in this laboratory (18). We have found that these virions contain approximately 15 to 20 different species of tRNA-like molecules and 5 and 7S RNAs, in agreement with the work of others (16, 31). Not all of the different species of RNA are associated with HMW-RNA. One of the smallest RNAs, which can act as a primer for initiation of RNA-directed DNA synthesis (5), is present in great molar excess over all other virion RNAs.

MATERIALS AND METHODS

Preparation of ³²P-labeled RNA. Cultures of chicken embryo fibroblasts (negative for avian leukosis virus group-specific antigen and RNA, and for chicken helper factor; E. Humphries and H. M.

Temin, personal communication) infected with SRD-RSV were obtained from Howard Temin and grown (1, 10) in plastic culture plates (60 mm diameter). Four days after infection, the culture medium was removed and replaced with 4 ml per plate of phosphate-free modified Eagle minimal essential medium containing 0.1 mCi of $^{32}\text{PO}_4^{3-}$ per ml and supplemented with 1% fetal bovine serum. After 48 h of phosphate labeling, the medium was removed, fresh $^{32}\text{PO}_4^{3-}$ -containing medium was replaced, and labeling was continued for an additional 24 h. RNA was prepared immediately from each harvest individually, without pooling. The purified RNAs were then pooled.

Culture medium containing the virus was chilled on ice immediately after removal from the culture plates. All subsequent virus purification steps were done at 0 to 4 C. Cellular debris was removed by centrifugation in a Sorvall GSA rotor for 10 min at 8,000 rpm. Virus was pelleted by centrifugation for 1 h at 26,000 rpm in a Beckman SW-27 rotor. The virus pellet was then resuspended in cold, nonradioactive Eagle minimal essential medium, layered on a 4-ml linear 10 to 65% sucrose gradient in TSE buffer (TSE = 0.02 M Tris-hydrochloride, pH 7.2; 0.1 M NaCl; 0.001 M EDTA), and centrifuged at 50,000 rpm for 30 min in a Beckman SW-50.1 rotor. This gradient was fractionated from the bottom, and the virus band was located by counting samples of each fraction in a planchet counter. The fractions containing the virus peak were pooled and diluted to 1.5 ml in TSE buffer. Carrier RNA (100 μg ; phenol-extracted Sigma type IV yeast RNA) and 1.5 ml of Pronase (Calbiochem, 500 $\mu\text{g}/\text{ml}$ in 0.01 M Tris-hydrochloride, pH 7.5, 0.014 M NaCl, predigested for 2 h at 37 C) were added (19). The preparation was incubated at room temperature for 10 min, and then 0.15 ml of 10% sodium dodecyl sulfate was added and incubation was continued at room temperature for an additional 20 min. This mixture was extracted twice with phenol saturated with TSE buffer and twice with chloroform-isoamyl alcohol (24:1). The RNA was then precipitated overnight at -20 C after addition of a one-tenth volume 2 M sodium acetate and 2 vol of ethanol.

In some experiments virion RNA was fractionated on sucrose gradients before polyacrylamide gel electrophoresis. HMW-RNA and the nonassociated small RNA fraction (4 to 7S) were separated in a 5-ml, 5 to 20% sucrose gradient in TSE buffer in a Beckman SW-50.1 rotor centrifuged for 1 h at 50,000 rpm. The purified 70S fraction was collected and pooled. (Fig. 1). The pooled fractions were precipitated with ethanol, as above, for further use.

In one experiment, 70S RNA was heated in TSE buffer to 95 C for 2 min, layered on an 18-ml, 5 to 20% sucrose gradient in TSE buffer in a Beckman SW-27 rotor, and centrifuged for 16 h at 25,000 rpm to yield 35S RNA and associated small RNA (4 to 7S). In another preparation, unfractionated RNA was heated to 95 C for 2 min and separated on an 18-ml gradient, as for 70S RNA, to yield total small RNA.

Two-dimensional polyacrylamide gel electrophoresis. The small RNAs of Rous sarcoma virus were separated from each other by a two-dimensional polyacrylamide gel electrophoresis (2-D gel) tech-

nique (18). All gels were run in an EC-470 cell (E-C Corp., St. Petersburg, Fla.). The first dimension was electrophoresed for 3 h at 400 V, 15 C, in a slab of 10% acrylamide (9.5% acrylamide, 0.5% *N-N'*-methylene-bisacrylamide). The samples were applied in melted agarose to a 1- by 4-mm slot as discussed below. The buffer was a one-half dilution of TEB buffer of Peacock and Dingman (22) (final concentrations: 0.045 M Tris base, 1.4 mM EDTA, 0.045 M boric acid; pH 8.3). During electrophoresis in the first dimension, bromophenol blue dye was added approximately every 45 min to aid in locating the RNAs. A strip of gel approximately 10 by 1 cm, containing the small RNAs, was cut out and placed in the EC cell at the origin, perpendicular to the original direction of electrophoresis. Twenty percent acrylamide (19% acrylamide, 1% *N-N'*-methylene-bisacrylamide) was poured around it. After polymerization the gel was run for 16 h at 400 V, 15 C with $0.5 \times$ TEB buffer.

FIG. 1. Sucrose gradient centrifugation of Rous sarcoma virus RNAs. Unfractionated viral RNA was separated into high-molecular-weight RNA and free small RNA in a 5-ml, 5 to 20% linear sucrose gradient in TSE buffer (1 h, 50,000 rpm, 4 C, Beckman SW-50.1 rotor). Sedimentation was from right to left. Fractions (0.25 ml) were collected from the bottom of the tube, and 1- μliter samples of each fraction were counted directly in a planchet counter. Fractions 1 through 13 were pooled for HMW-RNA (70S and associated small RNA). Fractions 20 through 23 were pooled for free small RNAs.

RNA was applied directly to the gel in a 60/40 mixture of buffer and warm (37°C) 2% agarose which solidified in the sample slot. Application of the sample in agarose led to smaller spots on the gel, and thus better resolution. In addition, since only RNA molecules about 300 nucleotides long or smaller entered a 10% polyacrylamide gel, the larger RNAs were trapped at the origin by the agarose for quantitation. The difficulty with agarose is that it tends to gel at about 35°C, so one must work quickly in loading the sample.

The autoradiograph of the gel was used to locate individual RNA species. Spots were cut out of the X-ray film with cork-borers, and the film was placed over the gel. The area of the gel containing each RNA was cut out by using a sterile plastic drinking straw of appropriate size and was placed in a counting vial. The gel pieces were crushed with a glass rod and radioactivity in each spot was counted by Cerenkov radiation (7) in 2 ml 0.3 M NaCl. After addition of 100 µg of carrier RNA, the RNA was eluted in the same salt solution by shaking at room temperature for 1 to 2 h. The eluted RNA was freed from the crushed gel by filtration through a membrane filter (Millipore Corp., HAWP 02500) on a Swinnex filtration base into a glass centrifuge tube. The filter was washed with two 0.5-ml samples of 0.3 M NaCl, and the filtrate was then precipitated overnight with 2 vol (6 ml) of ethanol. Control experiments showed that efficiency of counting was affected less than 2% by counting the radiation while the RNA was still in the gel.

Fingerprint analysis. After ethanol precipitation, the individual species of RNA were digested with RNase T1 (EC 2.7.7.26, Calbiochem, Sankyo; enzyme:RNA = 1:20) at 37°C for 30 min and fingerprinted according to the procedures of Sanger et al. (27).

RESULTS

Polyacrylamide gel electrophoresis. Separation of small RNAs of RSV by one-dimensional electrophoresis in 10% polyacrylamide gel is shown in Fig. 2. The locations of the 5 and 7S molecules, which have been described by others (4, 16, 21), are indicated in the margin. The HMW-RNAs were too large to enter the gel and are located at the top, immobilized in agarose. The figure shows three different samples of the same preparation of RNA. Sample a was total virion RNA which was run directly after isolation and ethanol precipitation; since this RNA was kept at or below 37°C through the preparation, we call RNA that entered this gel free small RNA. Sample b was the same as sample a except that the RNA was heated to 95°C for 2 min just before loading onto the gel; we call RNA that entered this gel total small RNA. Sample c was RNA that was first purified as a 70S RNA complex in a sucrose gradient (Fig. 1) and then heated to 95°C for 2 min just before loading; we call RNA in this sample that entered the gel

associated small RNA. Clearly, all three patterns are quite similar, although some differences in intensity of particular regions do exist. All three samples contain both 7S RNA and 5S RNA in addition to 4S RNA.

Although one-dimensional gels are adequate for purification of 5 and 7S RNAs, it is necessary to use 2-D gels for analysis of the 4S region. The two-dimensional separation was accomplished by cutting out the strip of gel indicated by the arrows (a separate strip for each sample) and placing the strip in an electrophoresis cell at the origin of a 20% polyacrylamide second dimension as discussed in Materials and Methods. The patterns of RNA species that were obtained are shown in Fig. 3. The added dimension in 20% polyacrylamide separated several RNAs which were not separated in the 10% dimension; likewise, the 10% dimension separated several molecules which had similar mobilities in 20% polyacrylamide. A total of 12 separate major spots can be distinguished in Fig. 3a and b (free and total small RNAs), whereas only seven major spots are visible in Fig. 3c (associated small RNA). As we shall discuss below, many of the spots seen in these autoradiograms represent pure species of RNA, as assayed by T1 RNase fingerprinting (27).

The 2-D gel patterns shown in Fig. 3a and b (free and total small RNA, respectively) are quite similar. This similarity was expected since approximately 75% of the small RNAs of RSV are free rather than associated. The pattern of RNAs which were associated with the 70S RNA (Fig. 3c) is superficially rather similar to those of the free and total small RNAs, but the patterns are not identical. The differences between the patterns were confirmed when RNAs from all spots shown in Fig. 3a, b, and c were eluted and characterized by T1 RNase fingerprinting (see below). Several RNAs were free but not associated; in contrast, all RNAs which were associated with the 70S RNA were also free. The distribution of the different RNA species is diagrammed in Fig. 3d, in which molecules which were only free are shown as open circles and molecules which were both free and associated are shown as filled circles. For reference, arrows on the 2-D gels indicate the positions of 5S RNA and spot 1, a major 4S RNA.

T1 RNase fingerprinting. Individual species of RNAs which were purified by the 2-D gels shown in Fig. 3 were analyzed by T1 RNase fingerprinting after elution of the RNA from the gel. A fingerprint is a very useful assay for establishing the purity of an RNA preparation or for testing whether two species with similar gel mobilities are identical. In Fig. 4 we present

FIG. 2. Separation of RSV small RNAs by one-dimensional electrophoresis in 10% polyacrylamide gel. a, Free small RNAs; b, total small RNAs; c, HMW-RNA associated small RNAs. The preparation of the RNAs is described in Materials and Methods. Samples (80×10^3 to 170×10^3 counts per min; see Table 1) were applied to the gel in melted agarose at 37 C and electrophoresed for 3 h at 400 V and 15 C. The figure is an autoradiogram of the ^{32}P -labeled RNAs in the gel. The location of HMW-RNA, 7S, and 5S RNAs are indicated in the margin; the positions of two 4S RNA species, spots 1 and 6, are also shown. The arrows indicate the portion of each first dimension strip used in the second dimension (see Fig. 3).

FIG. 3. Two-dimensional polyacrylamide gel electrophoresis patterns of small RNAs of Rous sarcoma virus. *a*, Free small RNAs; *b*, total small RNAs; *c*, HMW-RNA associated small RNAs; *d*, schematic drawing indicating the location of individual RNAs in the 2-D gel and the numbering system used here. Strips of first-dimension gels, as indicated in Fig. 2, were used as the origin for the second-dimension gels (20% polyacrylamide, 16 h, 400 V, 15 C) as described in Materials and Methods. The first dimension was from top to bottom; the second dimension was from right to left. The arrows indicate the major 5S RNA molecule and spot 1, a major 4S RNA species. Closed circles in the schematic drawing indicate species found both free and associated with the HMW-RNA. Open circles show species found only free.

only a representative sampling of the fingerprints, all of which were made on RNAs isolated from the free RNA preparation. The molecule from each gel spot in each preparation was identified by fingerprinting. For comparison, a fingerprint of unfractionated free small RNA, purified by sucrose gradient centrifugation (Fig. 1), is included in the figure. The RNAs from several gel spots such as 1, 2, and 6 have quite simple fingerprints, with a complexity that is usually found for molecules which are the size of tRNA (usually about 15 T1 RNase oligonucleotide spots). We interpret that simplicity as an indication that these particular RNAs are pure. Other RNAs, such as those isolated in spot 1b, have fingerprints which are more complex than one would expect for a single species; this

complexity indicates that spot 1b contains a mixture of several molecules.

In a number of cases, such as spot 5, the fingerprint of the RNA from the free sample is considerably more complex than that from the associated sample; however, all of the oligonucleotides in associated spot 5 are also present in the free spot 5. This result indicates that there are at least two types of RNA with similar electrophoretic mobilities, one of which is only free and the other of which is both free and associated with 70S RNA. The associated spot 5 RNA itself seems to be a mixture of two molecules. One of these associated spot 5 RNAs appears to be very similar to spot 6 RNA since, with only one exception, the spot 6 oligonucleotides constitute a subset of associated spot 5

oligonucleotides; the one exception is that an oligonucleotide in spot 6 RNA (arrow in Fig. 4, spot 6) is displaced slightly to the left in the fingerprint of spot 5 RNA. Thus spot 6 RNA appears to migrate both as a separate spot and with spot 5.

The RNA from each spot shown in Fig. 3d has a characteristic and reproducible fingerprint. Many of those RNAs are undoubtedly the tRNAs which have been described by several groups of workers. From the gel patterns shown in Fig. 3 and fingerprints such as those in Fig. 4, we estimate that approximately 9 associated 4S RNAs and an additional 6 free 4S RNAs can be detected by this method. Moreover, several other RNA spots can be seen upon longer exposure of the gel, but these molecules are present in amounts which are too low for fingerprinting.

Fingerprints of particular RNAs. The 7S RNA band shown in Fig. 2 was also eluted and fingerprinted. A T1 RNase fingerprint of this RNA isolated from sample a (free small RNA) had the same pattern of oligonucleotides as that published by Faras et al. (16), so it is not included in Fig. 4. Sample c of Fig. 2, the associated small RNA, also had RNA which co-electrophoresed with 7S RNA. When that RNA was eluted and fingerprinted, we found all of the oligonucleotides of the free 7S RNA, plus several others. From that result, we conclude that associated 7S RNA is probably more contaminated with HMW-RNA degradation products than is the free 7S RNA. The significant fact is that we did find some 7S RNA in the associated small RNAs, in contrast to the reports of others in studies of RSV (16). 7S RNA has been found associated with the 80S RNA of mouse L-cell virions (20).

We detected two forms of 5S RNA (spots 5S and 5S* in Fig. 3d) which had very similar fingerprints. The fingerprints are the same as those published elsewhere for 5S RNA (16). Since the amount of spot 5S* was greatly reduced in samples which had been heated, we feel that the two forms were conformational isomers of each other. Similar conformational isomers of 5S RNA have been detected for both the *Escherichia coli* and KB cell molecules (2, 17, 18).

The RNA in spot 1 (Fig. 3) was investigated more extensively than the others since it was the most abundant species of small RNA in either the free or the associated fractions. The fingerprint of this RNA (Fig. 4, spot 1) is almost identical to that published by Bishop et al. (5) for a molecule which serves as a primer for in vitro RNA-directed DNA synthesis, using the purified virion-associated DNA polymerase and

RSV RNA. More recent experiments carried out jointly in Bishop's, H. M. Goodman's, and our own laboratories confirm that the RNA molecule in spot 1 can be a primer (manuscripts in preparation). This molecule is rather short compared with other small RNAs, being 68 to 75 nucleotides long. It is quite similar to tRNA in that it has pGp and pCpCpA_{OH} at its 5' and 3' ends, respectively. In addition, it contains several modified nucleotides such as dihydrouridylic acid, pseudouridylic acid, and several methylated bases. We find that the free and the associated spot 1 RNAs have identical fingerprints and very similar, if not identical, minor nucleotide compositions.

Quantitation. The various small RNAs are not present in equal amounts, as can be seen in Fig. 3a, b, and c. An estimate of the number of each small RNA in both the free and the associated fractions can be made if one assumes that the specific activities of the RNAs are all approximately equal (11), that one 70S RNA complex of 30,000 nucleotides (12, 24, 28) exists per virion, and that there is no preferential loss of particular RNAs during purification. This estimate was made by cutting out the regions of the gels which contained the various molecules. The radioactivity in each piece of gel was determined by Cerenkov radiation counting before elution and fingerprinting. All radioactivity was normalized for the estimated size of the particular RNA molecule (judged by mobility in 10% polyacrylamide, using spot 1 and 5 and 7S RNAs as markers for 70, 120, and 250 nucleotides, respectively).

The quantitative data are presented in Table 1. It is clear that there is a wide range in the relative abundances of the various RNAs. In both the free and associated fractions, spot 1 predominates; in the free RNA, it is present approximately 6 times per virion, and in the associated fraction it is present 1.5 or 2 times per virion (two different experiments). Since several of the spots have been shown to contain mixtures of two or more RNA molecules (Fig. 4), the average numbers of molecules per HMW-RNA are the sums of the actual numbers for individual species. Thus, the number of free spot 5 RNAs per HMW-RNA must be divided by at least a factor of 3 since it contains three different RNAs which do not happen to separate in the 2-D gel. With this consideration in mind, it becomes clear that very few small RNAs are present as often as once per virion; these are spots 1, 2, 6, and 7S RNA and perhaps one or two molecules in the spots 4 and 5 region.

We have calculated the relative distributions of RNAs in two different preparations of RNA.

FIG. 4. Fingerprints of individual free small RNAs purified by 2-D gel electrophoresis. Individual RNA species were eluted from the 2-D gels, precipitated in ethanol, and digested with T1 RNase for fingerprinting according to the procedures of Sanger et al. (27). The first dimension was right to left on cellulose acetate, pH 3.5 pyridine acetate in 6 M urea. The second dimension was from top to bottom on DEAE-cellulose paper in 7% formic acid. The number of each fingerprint except T is from the 2-D gel schematic diagram (Fig. 3d). All fingerprints illustrated are from free small RNAs. T is a fingerprint of an unfractionated small RNA sample

FIG. 4.—Continued

which was separated from HMW-RNA by sucrose gradient centrifugation (Fig. 1). The thin vertical lines are radioactive ink markers. The dots on the middle right-hand side of each fingerprint denote the position of the blue dye marker (xylene cyanol FF). Arrow in fingerprint 6 denotes an oligonucleotide which is discussed in the text.

TABLE 1. *Numbers of small RNAs per virion^a*

Gel spot	Assumed no. of nucleotides	Free molecules/virion		Associated molecules/virion		Total molecules/virion	
		Counts/min	No.	Counts/min	No.	Counts/min	No.
HMW-RNA	30,000	116,000	1.0	80,000	1.0	160,000	1.0
7S	250	1,700	1.8	1,300	2.0	2,500	1.9
5S*	120	100	0.3				
5S	120	150	0.2	90	0.4	500	0.7
13	68	Not counted	Not counted		0.2	170	0.4
9	68	150	0.5			220	0.6
8	76	120	0.3	50	0.2	200	0.5
7	85	210	0.5	90	0.3	440	0.9
6	87	540	1.6	70	0.2	820	1.7
5	77	620	2.0	140	0.6	1,600	3.9
4	74	750	2.5			2,000	5.0
3	73	290	0.9	100	0.3	550	1.4
2	72	600	2.1			lost	
1b	69	430	1.6			230	0.6
1	68	1,700	6.4	290	1.5	2,400	6.4

^a Radioactivity in individual gel spots was determined by Cerenkov counting of the spots before elution of the RNA. Spots were located and cut out as described in Materials and Methods. Chain length of each molecule was based upon the data of others and by interpolation of the mobilities in 10% polyacrylamide relative to 7S, 5S, and spot 1 RNAs which were assumed to be 250, 120, and 70 nucleotides, respectively. This number of phosphates per RNA molecule was then divided into the counts per minute obtained for the respective gel spots, in order to normalize for chain length. The resulting ratio, counts per minute per nucleotide, is proportional to the number of copies of each RNA in the preparation. We assumed that there was 1.0 copy of HMW-RNA with 30,000 nucleotides per virion; therefore the numbers of the other RNAs were determined relative to 1.0 HMW-RNA by dividing the counts per minute per phosphate of individual spots by counts per minute per phosphate of HMW-RNA. HMW-RNA was defined as any RNA which does not enter 10% polyacrylamide gel; this includes 35S genomic RNA (and any small RNA which might be associated with it), and 18 and 28S RNAs. No attempt was made to apply equal amounts of radioactive RNA to the gel. RNA was prepared from virus which was harvested on days 2 and 3 after addition of ³²PO₄³⁻ to transformed cells (6 and 7 days after infection) to insure that specific activities of the RNAs would be equal (11). Quantitation of total spot 2 was not included because the sample was contaminated during elution. The number of free spot 13 molecules was not determined because of high background in that part of the gel; the number of associated spot 13 molecules was determined only once.

Results of the repeat experiment agree within a factor of 2 with the results reported here. Other experiments, using RNA preparations in which the 70S RNA was partially broken down, yielded numbers which indicated more small RNA per virion. This probably resulted from a reduction in the amount of radioactivity in HMW-RNA, and an increase in the background in the small RNA region of the gel.

DISCUSSION

Virions of avian RNA tumor viruses contain a mixture of small (4 to 7S) RNAs, which constitute 15 to 30% of the virion RNA. The association of some of these small RNAs with the 70S genomic RNA has been used as an initial step in fractionation of these molecules. The mixtures of RNAs which were so obtained have been studied in other laboratories with regard to polyacrylamide gel electrophoresis mobility (3, 4, 13, 14, 16), aminoacylation activities (13, 25,

29-31), extent of nucleotide methylation (9, 15, 23, 26), and ability to serve as primer for DNA synthesis (5, 8, 16, A. J. Faras et al., *J. Mol. Biol.*, in press). Most of these studies were performed on mixtures of RNAs which contain both specific molecules and nonspecific degradation products of HMW-RNA.

2-D gel. We have used 2-D gel electrophoresis (18) to characterize individual small RNAs of Schmidt-Ruppin D strain of Rous sarcoma virus. The patterns of small RNAs have been reproduced with approximately the same relative yields in six different preparations. The technique permitted careful analysis of individual species of RNA, rather than the "4S" class. In addition, since the RNAs were labeled by ³²PO₄³⁻, we were able to analyze all such molecules and not just the RNAs for which there is a biochemical assay. For example, we directly quantitated the relative amounts of 7 and 5S RNAs, several tRNA-like molecules, and a primer for the virion RNA-directed DNA

polymerase activity. The technique did not, however, directly tell us which RNAs had particular biochemical activities. This method of study also separated the distinct RNA molecules from nonspecific degradation products which tended to form a light haze throughout the gel; the distinct molecules were seen as individual spots, and only a small amount (if any) of the background degradation material was associated with them.

We found that an initial fractionation of the RNAs could be made by separating 70S RNA-associated molecules (A. J. Faras et al., *J. Mol. Biol.*, in press) from free molecules, either by sucrose gradient centrifugation or by polyacrylamide gel electrophoresis. The associated small RNAs constitute a subset of the free small RNAs. That is, all small RNAs which are isolated associated with the 70S RNA are also found in the free small RNA preparation. In contrast, there are several small RNAs which are free only, and not associated with 70S RNA, as we isolate it. We wish to emphasize that this free versus associated small RNA separation does not necessarily reflect the state of the RNAs within the virions, since some molecules may either associate with, or disassociate from, the 70S RNA during isolation. Thus our classification of free or associated RNA is purely operational at this time.

The validity of fractionating free small RNAs from associated small RNAs directly on the gel was tested by analysis of RNAs which were separated by sucrose gradient centrifugation (Fig. 1). The 2-D gel pattern of free small RNA isolated from a sucrose gradient was the same as that seen in Fig. 3a (unpublished observations of R.C.S. and J.E.D.). This result indicates that the presence of HMW-RNA at the origin of the 2-D gel does not influence which small RNAs enter the gel. In another experiment, we tested whether application of RNA to the gel affects the affinity of associated small RNA for HMW-RNA. Sucrose gradient-purified 70S RNA which was not heated before application to the gel in agarose had very little RNA which migrated into the 10% gel during the standard electrophoresis; the RNA which did migrate into the gel without prior heat treatment did not form any distinct bands, only a faint haze in the autoradiogram, which we assume represents some degradation products.

T1 RNase fingerprinting. In addition to characterization by mobility in the 2-D gel, the RNAs were also studied by T1 RNase fingerprinting (27). This latter procedure was very useful in determining the purity of the RNA preparation as well as the possible identity of molecules coming from two different sources

such as different places on the gel, different kinds of preparation (free or 70S associated), or different viruses.

Quantitation of small RNAs in the virion.

About 15 to 20 different small RNA species can be detected by 2-D gel analysis. Most of these species are present in fewer than one copy per virion, whereas a few are present in more than one copy per virion. Addition of the numbers of copies per virion of small RNAs which are found in either fractional or multiple amounts leads to the conclusion that there are an average of 30 to 35 molecules of small RNA per virion. The normalization which was used to calculate these numbers (Table 1) is subject to some errors. These errors would primarily alter the amount of radioactivity that would be included as "high-molecular-weight RNA." For example, small RNA which is associated with the 70S RNA and 18 and 28S RNAs which are in virions (21) would contribute to radioactivity which does not enter the gel. In addition, the release of small pieces of degraded genomic RNA would lead to an underestimation of the amount of radioactivity in HMW-RNA. These considerations would, however, affect a large (and as yet inexact) number in only a relatively small way. We do not feel that the data in Table 1 are reliable to more than $\pm 25\%$.

Previous reports by others place the total number of 4S RNA molecules per virion at 30 to 100 (3, 4, 9, 11, 12, 24). These numbers are somewhat higher than the 30 to 35 molecules which we calculate for RNAs which were purified by 2-D gel analysis. From sucrose gradient centrifugation profiles, we calculate that about 15 to 20% of virion RNA is 4 to 7S; this corresponds to about 6,000 nucleotides or 80 4S RNAs. Our estimate of 5 to 10 molecules of small RNA associated with 70S RNA is also lower than the figures given by others, which are in the range of 12 to 20 (11, 14, 16). Our lower numbers do not result from incomplete cutting of the spots from the gel since re-exposure of the cut gel slabs showed that no radioactivity remained at the edges of the cut regions. The lower figures could be influenced by some reproducible error such as quantitation of the amount of radioactivity in HMW-RNA. We feel that the higher estimates may be biased by degradation products of HMW-RNA. These degradation products would be counted as small RNA in assays which have less resolution. However, in our system, they are effectively diluted out by being spread throughout the 2-D gel.

Previous reports of aminoacylation of RSV small RNAs indicated that specific tRNAs were included in that class of molecules. However,

because of the low efficiency of charging, it was not clear whether the 4S RNAs were primarily tRNA-like or primarily degradation products with a low level of tRNA contamination. Fingerprints of a mixture of RSV 4S RNA had tRNA-like features (14, 16). The reproducible pattern of small RNAs in RSV which we obtain on the 2-D gel represents compelling evidence that this group of molecules is specific rather than primarily composed of degradation products. The fingerprints of individual molecules indicate that many of these are indeed tRNAs. Since we are studying RNAs by $^{32}\text{PO}_4^{3-}$ labeling, we cannot determine, at this time, which RNAs account for which aminoacylation activities. However, the total number of purified RNA species correspond rather well to the number of aminoacylation activities that are free in the virion (31).

Spot 1 RNA. The RNA of spot 1 is one of the most interesting 4S RNAs of Rous sarcoma virus. It is present in the highest amounts, both in the free and the 70S-associated fractions. It is one of the smallest of the RNAs, being 68 to 75 nucleotides long. It has several characteristics of a tRNA such as pGp and pCpCpA_{OH} at the 5' and 3' ends, and several modified nucleotides (manuscripts in preparation); nevertheless, one of its main functions is probably not as a tRNA but as a primer for RNA-directed DNA synthesis. Evidence for this assertion is that spot 1 RNA has the same fingerprint as an RNA which serves as primer in vitro and which is the last small RNA to dissociate from HMW-RNA at elevated temperatures (5). Additional experiments which were performed jointly in Bishop's, H. M. Goodman's and our own laboratories have confirmed this conclusion (manuscripts in preparation).

Although we calculate that there are between six and nine molecules of spot 1 RNA per virion, only about two of these are associated with 70S RNA as we isolate it. We cannot say whether the figure of two is significantly different from the three or four molecules of 35S RNA found per virion. The T1 RNase fingerprints of the free and associated spot 1 RNAs are the same. Free spot 1 RNA molecules may have specific functions or they may be molecules which were released during preparation. Two possible functions of free spot 1 RNA could be (i) to serve as primer for initiation of discontinuous DNA synthesis at sites other than the 3' end of the template, or (ii) to prime the initiation of synthesis of complementary (+) strand DNA by using nascent DNA as template. Alternatively, the free spot 1 molecules may have no function at all. It is worth noting that since there are

several-fold more copies of spot 1 RNA than 35S RNA per virion, it is unlikely that this primer is derived by cleavage of the 35S template near its 3'OH end.

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