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Virion-Associated RNA Primer for Rous Sarcoma Virus DNA Synthesis: Isolation from Uninfected Cells

ROBERT C. SAWYER, FUMIO HARADA, AND JAMES E. DAHLBERG

Department of Physiological Chemistry, University of Wisconsin Medical School, Madison, Wisconsin 53706

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Uninfected chicken, duck, rat, and human fibroblast cells in culture contained a tRNA-like RNA molecule which was structurally identical to a virion-associated RNA primer for in vitro Rous sarcoma virus DNA synthesis. This primer RNA appeared to be a normal tRNA of these cells. It was not found in a number of lower eukaryotic cells or in *Escherichia coli*.

One of the 4*S* RNA molecules in Rous sarcoma virus (RSV) virions serves as the major (>85%) primer for RSV RNA-directed DNA synthesis in vitro, using either the purified virion-associated DNA polymerase and 70*S* RNA or detergent-disrupted virions (4, 6, 9, 15, 17). This RNA may well function as a primer in vivo, although that remains to be established. This molecule, which we call "spot 1 RNA," is the most abundant small RNA in the virion (26). It can be isolated from the other small RNAs of RSV virions by two-dimensional polyacrylamide gel (2-D gel) electrophoresis (20, 26), and it has been characterized by fingerprinting after T1 and pancreatic RNase digestion (4, 15, 26).

The experiments which we report here demonstrate that an RNA identical to spot 1 RNA can be isolated from both RSV-infected and uninfected chicken embryo fibroblast (CEF) cells and from uninfected duck, rat, and human fibroblast cells. These results indicate that spot 1 RNA is a cell-coded molecule.

MATERIALS AND METHODS

^{32}P labeling of cells in tissue culture. The following cell cultures were obtained from Howard M. Temin: uninfected and Schmidt-Ruppin-D-RSV-infected CEF cells, uninfected duck embryo fibroblast cells, B77 virus-infected pheasant and quail embryo fibroblast cells, and uninfected rat embryo fibroblast cells, NRK cells, and Coon's rat liver cells. Cultures of human fibroblast cells were obtained from Robert I. DeMars. Cells were labeled with $^{32}PO_4^{3-}$ for 48 h, as described elsewhere (1, 11, 26). After labeling, the cells were washed twice with 5 ml of cold buffer (0.02 M Tris-hydrochloride, pH 7.2; 0.1 M NaCl; 0.001 M EDTA) and gently scraped off the plates with a rubber spatula in 2 ml of buffer per plate. RNA was purified by phenol-sodium dodecyl sulfate extraction and ethanol precipitation.

^{32}P labeling of other cells. Drosophila (*Drosophila melanogaster*) larvae were grown in 125-ml wide-mouth bottles plugged with cotton. Each bottle contained 30 ml of unlabeled agar (1.8% agar, 10% glucose, 0.5% propionic acid, and 10% brewer's yeast) overlaid with 5 ml of the same agar containing 2 mCi of $^{32}PO_4^{3-}$. Approximately 100,000 drosophila eggs were placed on the agar surface and incubated for 8 days at 26 C. Second instar larvae were harvested by washing with water on a fine-mesh screen to remove agar and unincorporated $^{32}PO_4^{3-}$. RNA was prepared by phenol extraction and DEAE column chromatography according to the method of Twardzik et al. (28).

Neurospora were grown as follows. The culture was started by adding 20 ml of inoculum (strain 74-OR8-1a conidia, $OD_{420} \cong 0.4$, $\cong 2 \times 10^6$ conidia/ml) to an equal volume of medium containing 10 ml of Fries 4X PO_4-free salts, 0.6 g of sucrose, and 0.2 ml of 50 mM KH_2PO_4 (2). The culture was then shaken for 5 h at 30 C. After centrifuging the culture, 30 ml of supernatant medium were removed and discarded, 1 mCi of carrier-free $^{32}PO_4^{3-}$ was added, and the culture (final volume, 10 ml) was again shaken for 5 h at 30 C. The conidia were collected by filtering the culture through a membrane filter (Millipore Corp.) and were washed with water, and the RNA was prepared by phenol-sodium dodecyl sulfate extraction.

Physarum cells (*Physarum polycephalum* M_3C VII) were grown as mycoplasmodia in a 20-ml culture containing 1 mCi of $^{32}PO_4^{3-}$ according to the method of Daniel and Baldwin (10), except that total phosphate was 2×10^{-3} M. The culture was shaken at 26 C for 2 days. The mycoplasmodia were pelleted and resuspended, and the RNA was prepared by phenol extraction and DEAE column chromatography as for *Drosophila* RNA.

Normal tobacco (*Nicotiana tabacum* L. var. Wisconsin no. 38) and tobacco crown gall (22) were grown according to the conditions of Kemp and Sutton (21). Pith cylinders weighing approximately 150 mg were placed on 20 ml of agar medium containing 2 mCi of $^{32}PO_4^{3-}$ and allowed to grow at 28 C in the dark for 4 to 5 days (until mass was doubled). Agar medium contained, per liter: agar (10 g), sucrose (30 g),

thiamine (0.5 mg), myoinositol (100 mg), 11.5 mM indole-3-acetic acid, and 1 mM kinetin, 1.25 mM K_2HPO_4 (22). RNA was prepared as for *Physarum* and *Drosophila*.

Escherichia coli RNA was prepared as previously described (20).

Total virion RNA was isolated from purified Schmidt-Ruppin-D-RSV virions after $^{32}PO_4^{3-}$ labeling as described previously (26). The 2-D gel electrophoresis and fingerprinting were described elsewhere (20, 25, 26). Minor nucleotide analysis by thin-layer chromatography was performed according to the method of Nishimura (23).

RESULTS

Isolation of spot 1 RNA from uninfected chicken cells. We have previously shown that 2-D gel electrophoresis could separate the small RNAs of RSV into discrete species (26). In the present experiments, we used the 2-D gel to compare small RNAs from the virus with the small RNAs of a number of host and non-host cells. An autoradiogram of a 2-D gel electropherogram of total RSV small RNAs is shown in Fig. 1a. This is essentially the same pattern of RNAs which we reported previously (26). The position of spot 1 RNA is indicated by the arrow. This molecule has been identified as the major primer for in vitro RSV DNA synthesis (9, 15). A 2-D gel of small RNAs isolated from uninfected CEF cells is shown in Fig. 1b. A 2-D gel pattern identical to that in Fig. 1b was obtained when RNA from RSV-infected chicken cells was examined. A cellular molecule which has the same mobility as spot 1 RNA is indicated by the arrow. Figure 1c shows the numbering system for the CEF RNAs to which reference is made later (Table 1).

Fingerprint analysis of host cell spot 1 RNA. RNase T1 fingerprints of the virion and cellular RNAs, which are indicated by the arrows in Fig. 1, are shown in Fig. 2a and b. The similarity in the patterns indicates that the two RNAs have the same sequence. As we discuss below, corresponding oligonucleotides from the two fingerprints are present in equimolar amounts (Table 2) and produce identical products when further digested with pancreatic RNase. Compositions of the viral RNA oligonucleotides are presented elsewhere (15). Likewise, fingerprints made after pancreatic RNase digestion of both RNAs are identical (Fig. 2c and d). T2 RNase digestion of individual oligonucleotides followed by thin-layer chromatography (23) reveals that both the viral and CEF spot 1 RNAs contain the same modified nucleotides (15; F. Harada, R. C. Sawyer, and J. E. Dahlberg, manuscript in preparation). We conclude that spot 1 RNAs from the two sources probably have the same structure.

Yields of spot 1 RNA in uninfected versus infected CEF cells. The relative yields of spot 1 RNA in RSV-infected and uninfected CEF cells were determined by cutting out the gel spots as indicated in Fig. 1c and counting radioactivity in each spot before elution and fingerprinting (26) (Table 1). After analyzing several preparations, we conclude that spot 1 RNA is present in about the same amount relative to 5*S* RNA and most other 4*S* RNAs in both uninfected and RSV-infected transformed CEF cells.

Endogenous viral sequences. The significance of the observation that spot 1 RNA is present in uninfected cells requires that the cells really be "uninfected." Several control experiments were performed to test this assertion. Spot 1 RNA was isolated in approximately the same yield when extracts were made from independently established uninfected fibroblast cell cultures from two different chicken embryos. However, all chicken cells so far studied do contain some RSV nucleic acid sequences (29). Unpublished results of E. Humphries and H. M. Temin showed that both of the chicken cell cultures which we used gave negative results when tested for avian leukosis virus functions such as the avian leukosis-sarcoma virus group-specific antigen and chicken helper factor (18, 19, 31). The cells did contain a small amount of RNA which hybridized to RSV-RAV-O DNA (8, 19); with RNA from the two chicken cell cultures used at a C_rt of 1.7×10^4 and 7.8×10^3 mol-s/liter, the cell RNA protected 5.8 and 5.1% of a RSV-RAV-O viral DNA probe, respectively (E. H. Humphries and H. M. Temin, personal communication). The significance of any protection at this low level is not known.

Spot 1 RNA is labeled at the same rate as other small RNAs. In view of the unusual nature of this molecule (i.e., a tRNA-like molecule which serves as a primer for viral DNA synthesis [9, 15]), it was important to determine whether the rates of synthesis and degradation differed from those of other cellular stable small RNAs. Both RSV-infected and uninfected CEF cells were labeled with $^{32}PO_4^{3-}$ for 1, 4, 8, 12, 16, and 24 h and for 24 h followed by a 24-h chase with cold phosphate-containing medium. The amount of spot 1 RNA relative to other small RNAs remained constant (±25%) at each time point (data not shown). We conclude that in RSV-infected and uninfected chicken cells, spot 1 RNA is neither preferentially synthesized nor degraded relative to other small RNAs.

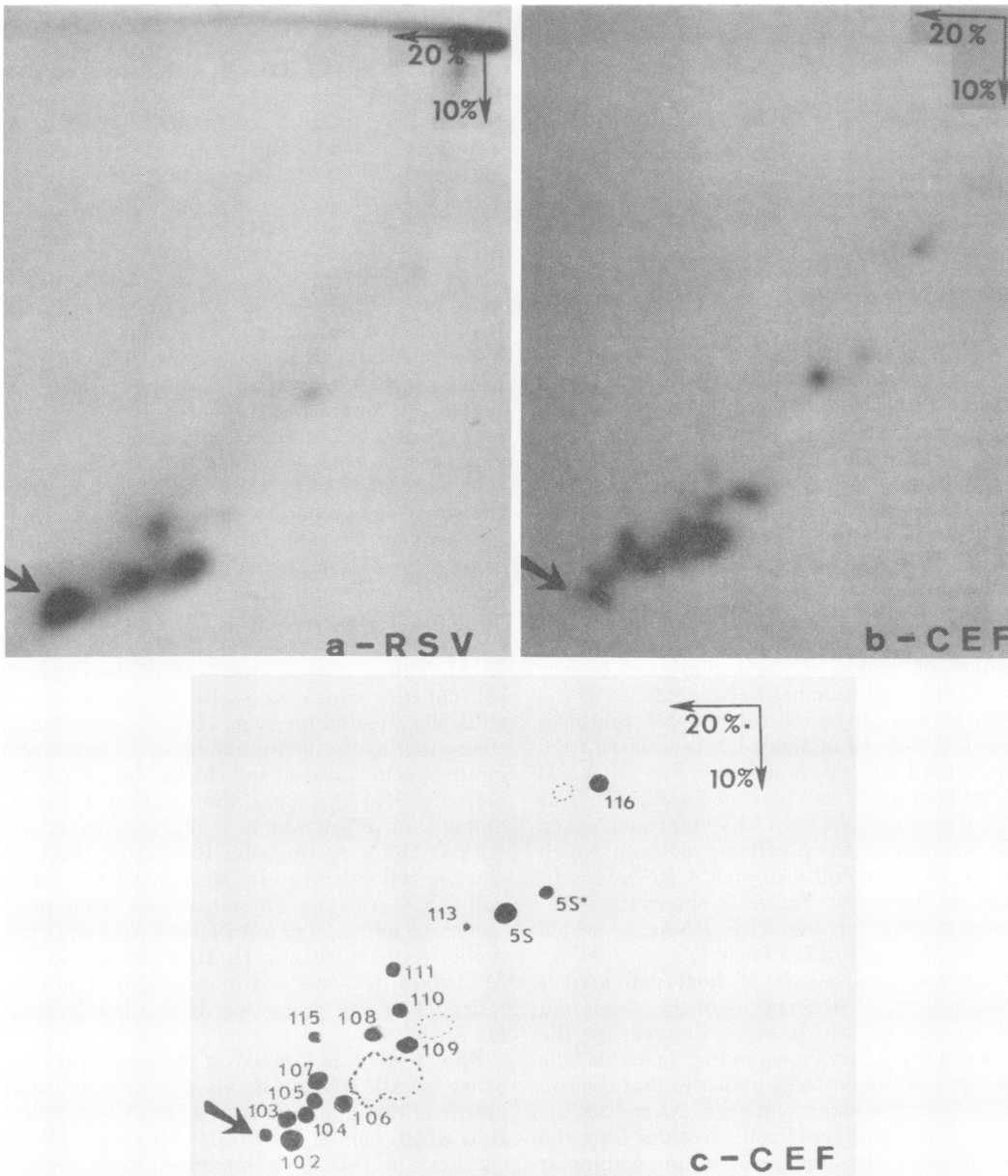

FIG. 1. *Two-dimensional polyacrylamide gel patterns of the total small RNAs from (a) Rous sarcoma virus virions and (b) uninfected CEF cells. (c) Numbering system for chicken cell small RNAs: the dashed lines outline RNAs which were not counted (see Table 1). The first dimension was 10% polyacrylamide (top to bottom), and the second dimension was 20% polyacrylamide (right to left). The arrows indicate the position of spot 1 RNA. 5S and 5S* are conformational isomers of 5S RNA (26). The autoradiographs were exposed for 4 h (a) and 15 min (b).*

Isolation of spot 1 RNA from other avian cells. To test whether information of endogenous avian leukosis-sarcoma viruses coded for the synthesis of spot 1 RNA, we studied the small RNAs of other avian cells, in particular, fibroblast cells from Muscovy duck embryos. Varmus et al. (29) showed that Pekin duck cells contained little or no DNA that was complementary to a B77 virus DNA probe (representing at least 30% of B77 virus RNA sequences).

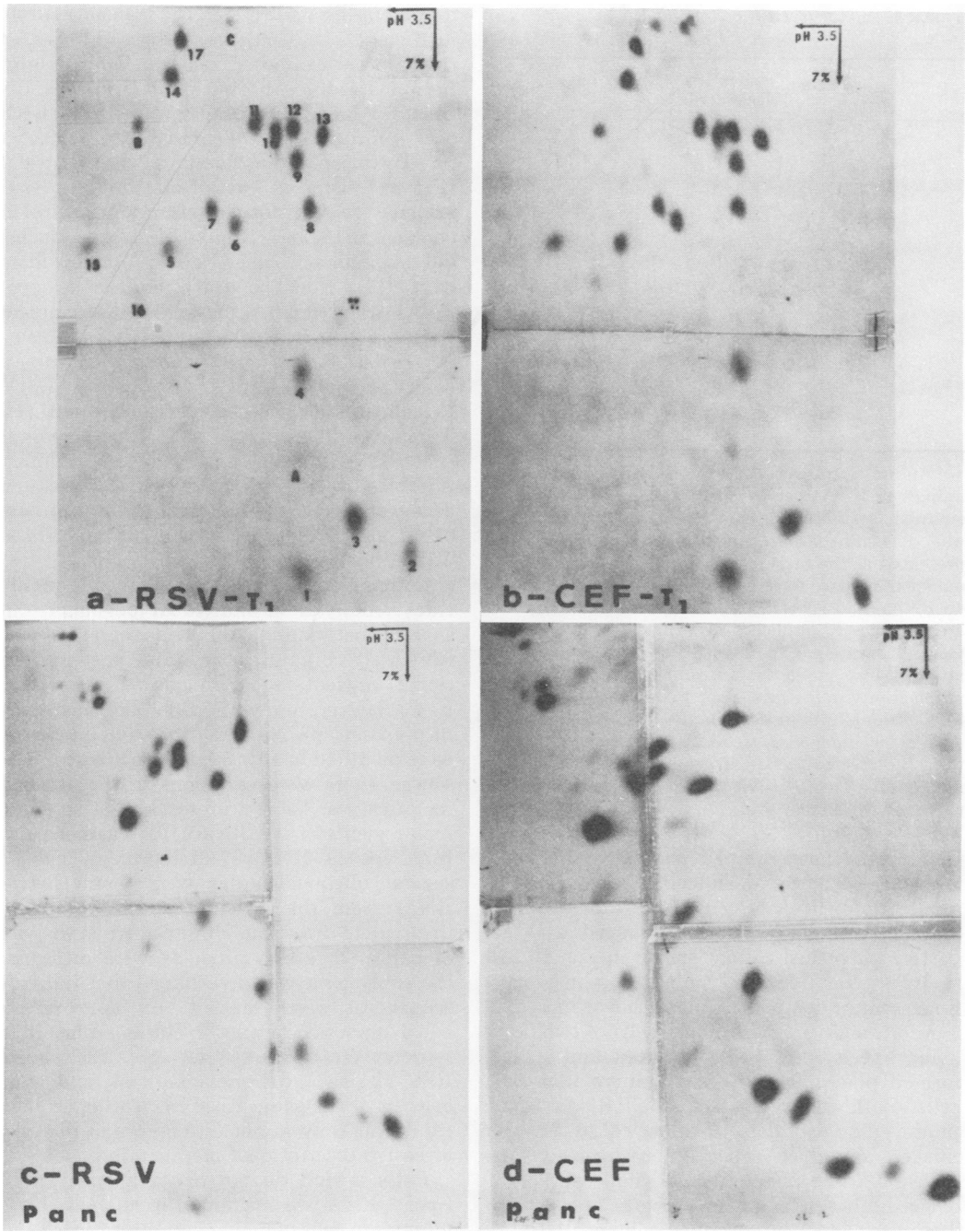

FIG. 2. *Fingerprints of spot 1 RNA isolated from virions of RSV (a and c) and uninfected CEF cells (b and d). Spot 1 RNAs were purified by 2-D gel electrophoresis (Fig. 1) and digested with RNase T1 or pancreatic RNase for fingerprinting according to the procedures of Sanger et al. (25). The first dimension was on cellulose acetate in pyridine acetate, 6 M urea, pH 3.5 (right to left). The second dimension was on DEAE-cellulose paper in 7% formic acid (top to bottom). (a) RNase T1 fingerprint of viral spot 1 RNA. (b) RNase T1 fingerprint of CEF cell spot 1 RNA. (c) Pancreatic RNase fingerprint of viral spot 1 RNA. (d) Pancreatic RNase fingerprint of CEF cell spot 1 RNA. UMP ran off the end of this particular fingerprint. The numbers in (a) are the numbering system used in Table 2 and reference 15.*

TABLE 1. *Yields of CEF cell small RNAs relative to spot 1 RNA*[a]

RNA spot No.	Uninfected chicken cells	Infected chicken cells
102	3.4	3.3
103	2.0	0.9
104	1.6	1.0
105	2.6	1.7
106	5.8	4.2
107	2.9	2.1
108	0.8	0.9
109	2.2	1.6
110	1.1	0.8
111	0.5	0.7
113	0.3	0.3
115	0.4	0.5
116	2.5	2.8
5S + 5S*	5.2	4.9

[a] Autoradiograms of the 2-D gels were used to locate individual RNA species (Fig. 1). Gel spots were cut out with a sterile drinking straw, and radioactivity was determined by Cerenkov counting. Data are presented as the ratio of radioactivity in the individual spots relative to that in spot 1. Spots 102–115 are 4S RNAs, and spot 116 is 5.5S rRNA. The numbers are averages of duplicate experiments in which the yields of particular spots varied as much as 30%. No attempt was made to apply constant amounts of radioactivity to the gels. Spot 1 contained 84,000 and 35,000 counts/min in two samples from infected cells, and 20,000 and 36,000 counts/min in two samples from uninfected cells. In some experiments the amount of 5S + 5S* RNA ranged as high as twice the values shown here.

C.-Y. Kang has obtained similar results with DNA of Muscovy duck cells (personal communication). RNA of cells from three different uninfected duck embryos produced 2-D gel patterns identical to that shown in Fig. 1b. All samples contained a molecule which gave fingerprints identical to those shown in Fig. 2.

Additionally, we examined cells of two other avian species, B77 virus-infected and -transformed pheasant and quail embryo fibroblast cells. Both cell types had small RNAs which produced 2-D gel patterns identical to those for chicken and duck cells. When digested with RNase T1, the pheasant and quail cell spot 1 RNAs both produced fingerprints identical to those from chicken and duck cell spot 1 RNAs (data not shown). We did not examine uninfected pheasant or quail cells. We conclude spot 1 RNA is probably common to all avian species.

Isolation of spot 1 RNA from rat cells. To determine whether spot 1 RNA existed in non-avian vertebrate cells, we looked for it in rat cells. Mammalian cells in culture can be transformed by RSV, although the transformation is inefficient (1). Cells from three different rat tissues were examined: NRK cells, rat embryo fibroblast cells, and a line of rat liver cells. RNA from all three cell types contained spot 1 RNA as determined by 2-D gel mobility (Fig. 3a) and fingerprinting after digestion with RNase T1 (Fig. 4a). The patterns of oligonucleotides obtained were very similar to that for the viral and chicken cell spot 1 RNAs (Fig. 2). Again individual oligonucleotides yielded products identical to those from the viral or chicken cell spot 1 RNAs when further digested with pancreatic RNase. The yields of spot 1 RNA relative to other small RNAs were the same in all three rat cell types.

Isolation of spot 1 RNA from human cells. These same techniques were used to identify spot 1 RNA in cells of two independent human fibroblast cell strains. Spot 1 RNA from human cells was essentially identical to that from cells of rat and avian species (Fig. 3b and 4b, and Table 2).

Comparison of spot 1 RNA oligonucleotides. Analysis of individual oligonucleotides showed that mammalian, avian, and viral spot 1 RNAs all contained the same post-transcriptional nucleotide modifications (F. Harada, R. C. Sawyer, and J. E. Dahlberg, manuscript in preparation). Although the same nucleotides were modified in spot 1 RNA isolated from each source, there were variations in the extents of modification. Table 2 presents a listing and the molar yields of the RNase T1 oligonucleotides from spot 1 RNAs. Differences in yields of several oligonucleotides were found between RNAs from the different species because of incomplete digestion. However, when the yields of partial digestion products were included in the analysis, it was determined that individual sequences were present in approximately equal amounts in spot 1 RNAs from all four sources. We conclude that spot 1 RNAs from RSV virions and from avian, rat, and human cells have the same basic nucleotide sequence, exhibiting only minor differences in the extent of post-transcriptional modification.

Cells which do not contain spot 1 RNA. Spot 1 RNA was not found in all cells. Having found it in cells of higher vertebrate species, i.e., avian, rat and human cells, we analyzed the small RNAs of several other eukaryotes and a bacterium. For each species, uniformly ^{32}P-labeled RNA was subjected to a 2-D gel electrophoresis, and each RNA in the general area of each gel where spot 1 RNA could be expected to migrate (arrows, Fig. 5) was cut out, eluted,

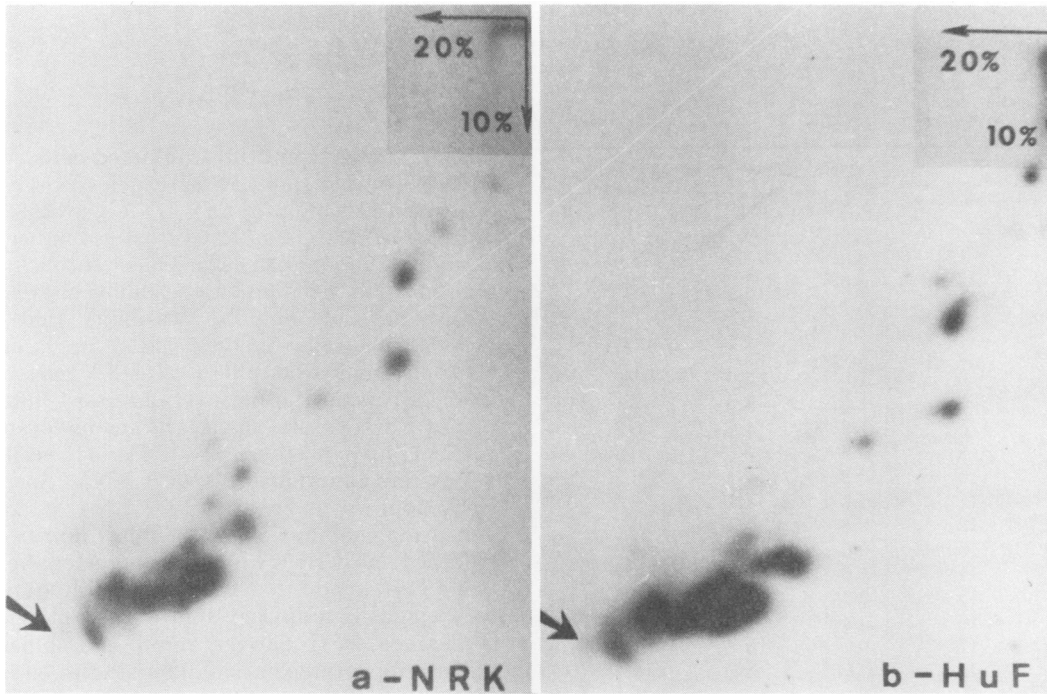

FIG. 3. *Two-dimensional polyacrylamide gel patterns of the total small RNAs from (a) NRK cells and (b) human fibroblast cells. The arrows indicate the position of spot 1 RNA.*

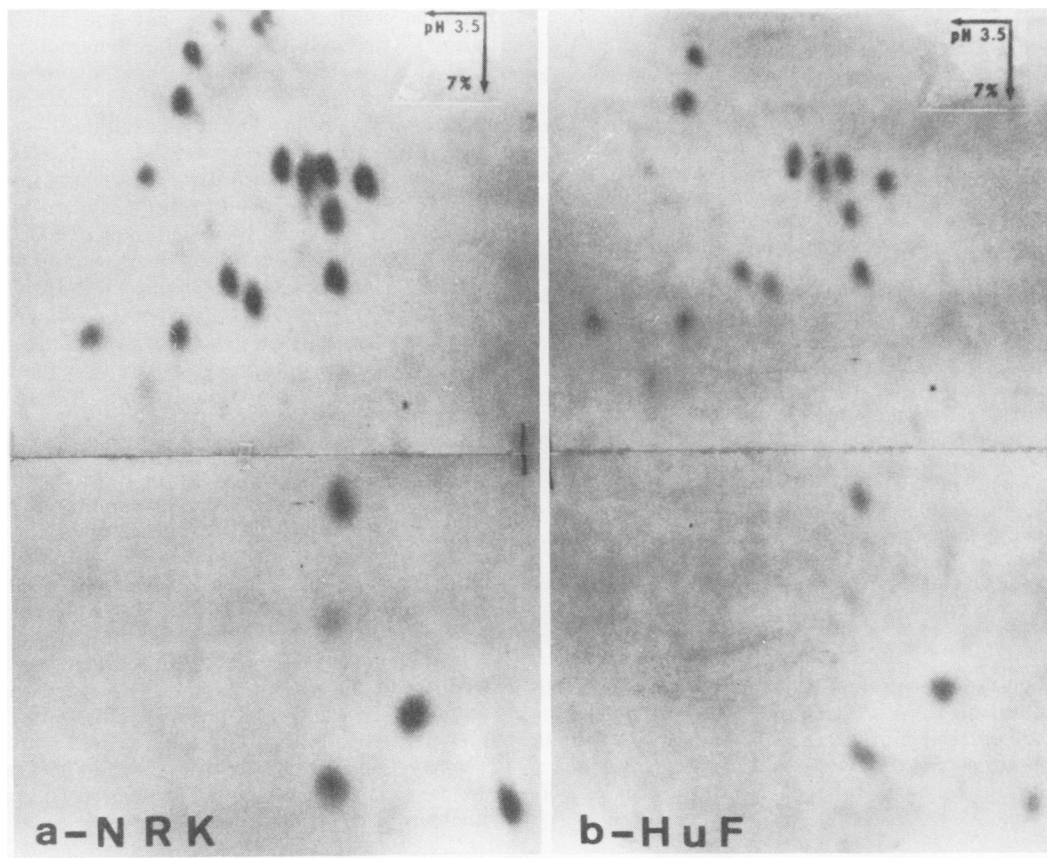

FIG. 4. *Fingerprints of spot 1 RNA isolated from (a) NRK cells and (b) human fibroblast cells. Spot 1 RNAs were isolated and fingerprinted after digestion with RNase T1 as described in the legend for Fig. 2.*

TABLE 2. *Molar yields of oligonucleotides obtained by digestion of spot 1 RNA with RNase T1*[a]

Oligonucleotide no.	Molar yield in primer isolated from:			
	RSV	CEF	NRK	HuF
1	4.9	4.7	3.4	5.4
2	1.0	0.9	1.0	1.1
3	3.1	2.7	2.8	3.0
4	1.0	1.0	1.0	1.0
5	1.2	1.3	1.2	1.3
6	1.0	1.0	1.0	0.9
7	1.0	1.0	1.1	1.1
8	1.2	1.2	0.8	0.8
9	0.8	0.6	0.6	0.3
10	0.9	1.1	0.9	1.0
11	1.1	1.1	0.8	0.9
12	0.8	0.9	0.7	0.7
13	0.6	0.6	0.6	0.6
14	1.6	1.5	1.1	1.3
15	0.9	1.0	0.3	1.3
16	0.4	0.5	0.5	0.6
17	0.7	0.8	1.0	1.0
B	ND	ND	0.7	0.3
C	0.2	(<0.1-0.3)	0.4	0.2
10 + 11 + 2C	2.4	2.3	2.5	2.3
14 + 17	2.3	2.3	2.2	2.3

[a] Spot 1 RNA was prepared by 2-D gel electrophoresis of RNA from RSV, CEF, NRK, and human fibroblast (HuF) cells. The RNA was digested with RNase T1 and subjected to 2-D paper electrophoresis (fingerprinting) according to the procedures of Sanger et al. (25). Individual oligonucleotides were cut out of the fingerprint, and radioactivity was determined in a gas-flow planchet counter. Molar ratios are relative to one copy each of the oligonucleotide numbers 4, 6, and 7, each of which is a trinucleotide. Radioactivity in each oligonucleotide was normalized for the number of phosphates contained in it, as deduced from its composition (15). Results are averages of the following numbers of determinations: RSV, 6; CEF, 4; NRK, 2; HuF, 2. Oligonucleotide A is ApGp and results from minor contamination in some preparations (15). Oligonucleotides B and C are partial digestion products. B contains oligonucleotide 16 plus an additional modified GMP. Oligonucleotide C contains oligonucleotides 10 and 11 and showed a large variability in yield. In some cases the yield was as much as 0.8 mol, with a concomitant decrease in the yields of oligonucleotides 10 and 11. Partial cleavage of oligonucleotide 17, which contains a modified GMP, produces ψpCpUpGp, which migrates with oligonucleotide 14 (15). ND, Not done.

digested with RNase T1, and fingerprinted. We found no RNA which produced an oligonucleotide pattern characteristic of spot 1 RNA in cells of *Drosophila* larvae, *Neurospora*, *Physarum*, normal tobacco, tobacco crown gall, or *E. coli* (Fig. 5).

DISCUSSION

Spot 1 RNA found in uninfected cells. We have shown that spot 1 RNA, which serves as a primer for initiation of RSV DNA synthesis in vitro (9, 15), can be isolated from a number of avian and mammalian cells. Thus, we conclude that spot 1 RNA is probably cellular in origin. We cannot rule out the possibility that in RSV-infected cells some spot 1 RNA might also be transcribed from the viral DNA genome; however, within the limits of our assay, infection by RSV does not change the amount of spot 1 RNA relative to other small RNAs of the cell.

Possible functions of spot 1 RNA. Spot 1 RNA appears to be a transfer RNA of both avian and mammalian cells. It has numerous modified nucleotides which are characteristic of tRNA (15). In addition, cellular spot 1 RNA can be specifically aminoacylated with tryptophan (F. Harada, R. C. Sawyer, and J. E. Dahlberg, manuscript in preparation). Our studies on the kinetics of labeling in RSV-infected and in uninfected CEF cells show that spot 1 RNA is a stable cellular RNA. The amount of spot 1 RNA relative to other small RNAs of CEF cells remains constant regardless of the labeling period. The striking tRNA-like character of spot 1 RNA (15) indicates that it probably serves in cellular protein synthesis, although additional functions cannot be excluded.

The fact that spot 1 RNA is present in uninfected chicken and duck cells raises questions about its possible role early in RSV infection, if, in fact, it is used as a primer for viral RNA synthesis in vivo. Utilization of host cell spot 1 RNAs may not be possible if DNA synthesis is initiated in a region of the cell where spot 1 RNA might be low (for example, in the nucleus). This would suggest a reason for the inclusion of six to ten copies of spot 1 RNA in the virion (26).

RSV can transform mammalian cells in culture (1). The presence of spot 1 RNA in uninfected rat and human cells is consistent with the possibility that it might serve as a primer in vivo in these cells.

Comparison of spot 1 RNA from several sources. Our analyses of spot 1 RNA from virions of RSV and from avian and mammalian cells suggest that spot 1 RNAs from these sources all have the same basic nucleotide sequence. However, we do note differences in the extents of some post-transcriptional modifications. The reasons for these differences are unclear but may reflect differences in the metabolism of the different cell types.

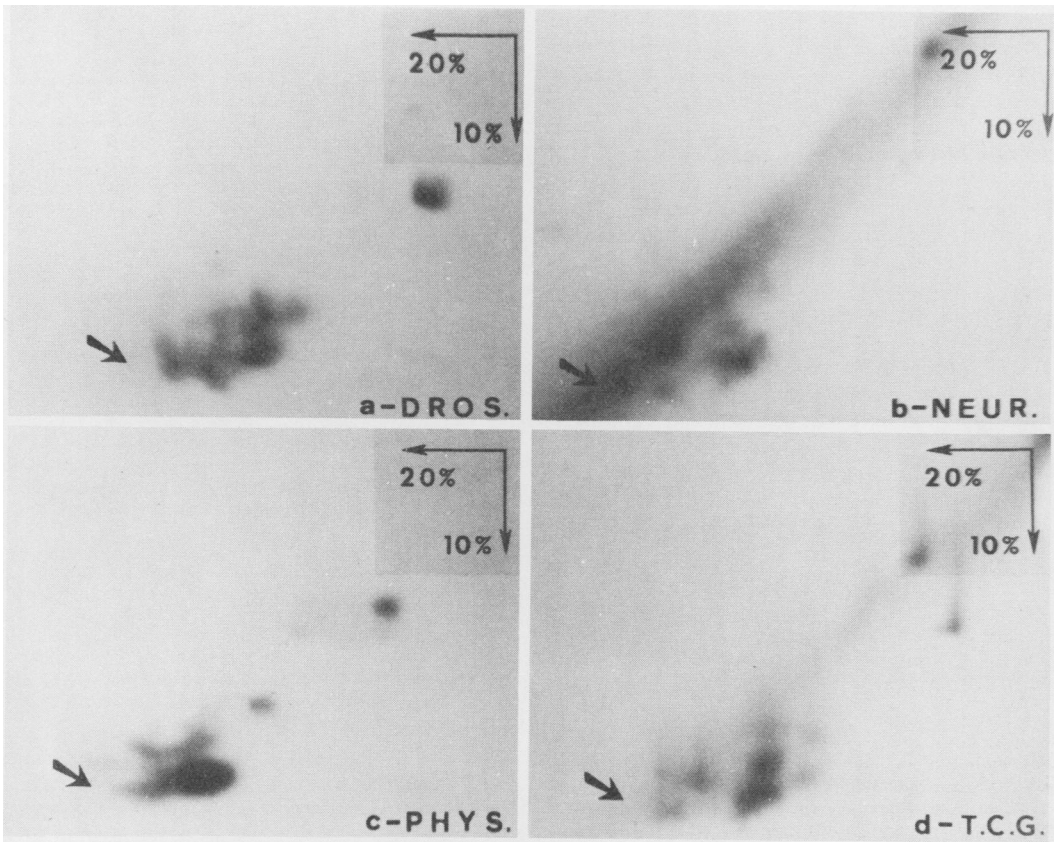

FIG. 5. *Two-dimensional polyacrylamide gels of small RNAs from (a) Drosophila larvae, (b) Neurospora, (c) Physarum, and (d) tobacco crown gall. RNA was prepared for each species as described in Materials and Methods. The 2-D gel pattern for E. coli small RNAs has been published elsewhere (20). The expected position of spot 1 RNA on these gels (arrow) was determined by comparison with gels of CEF RNA. All RNAs in that general area were eluted and fingerprinted as discussed in the text; no molecule was found which corresponded to spot 1 RNA.*

Spot 1 RNA not found in all cells. Some eukaryotic cells do not contain spot 1 RNA. We note that each species in which we found spot 1 RNA was labeled in tissue culture, whereas those species in which we were unable to find spot 1 RNA (except tobacco and tobacco crown gall) were labeled as whole organisms. We do not believe spot 1 RNA is an artifact of growing cells in tissue culture since we have recently found that spot 1 RNA can be isolated from chicken liver (F. Harada, R. C. Sawyer, and J. E. Dahlberg, manuscript in preparation).

We recognize that our inability to find spot 1 RNA in a number of species may be a reflection of different post-transcriptional modifications or minor changes in the basic nucleotide sequence which could alter electrophoretic mobility in the 2-D gel. However, in each of these preparations we analyzed all RNAs in the general area to which spot 1 RNA should migrate and found no molecule which produced a fingerprint resembling that for spot 1 RNA.

In some instances DEAE column chromatography was necessary to purify the RNA from several of these species (28) due to the large amount of carbohydrate which was not removed by phenol extraction and which precipitated with the RNA in ethanol. This carbohydrate caused the RNA to streak across the first dimension of the 2-D gel. Control experiments with naked CEF RNA showed that spot 1 RNA was not removed from bulk small RNA by this step. Therefore, we feel it is probable that spot 1 RNA does not exist in these particular cells.

Viral RNAs of cellular origin. Several other RSV virion RNAs are of cellular origin besides spot 1 RNA (3, 5), including 7*S* (14) and rRNAs (5, 16). In addition, RNA of cellular spot 116

(Fig. 1c), which has the fingerprint of 5.5S ribosomal RNA (16), is also present in virions, both free and associated with the 70S RNA complex (data not shown). The question of how specific cellular RNAs are included in the virion remains unanswered. Some of them may be in subcellular structures which are taken up during virion budding. Others may be taken into the virion by virtue of sequence complementarity with the high-molecular-weight virion RNA. It is not clear whether virion small RNAs serve any function during infection or transformation. From the work of others (3, 5, 7, 12, 13, 24, 27, 30) and from the different complexities of 2-D gel patterns in Fig. 1, it is clear that the RSV small RNAs are not a random sample of cellular RNAs. In particular, spot 1 RNA makes up a major proportion of the virion 4-5S RNA, whereas it is only a minor fraction of the chicken cell small RNA (compare Fig. 1a and b).

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