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# Low-Molecular-Weight RNAs of Moloney Murine Leukemia Virus: Identification of the Primer for RNA-Directed DNA Synthesis

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The small RNAs of Moloney murine leukemia virus (M-MuLV) were fractionated into at least 15 species by two-dimensional polyacrylamide gel electrophoresis. The pattern of small RNAs is significantly different from that of Rous sarcoma virus. A subset of the virion small RNAs is associated with the genome RNA in the 70S complex. One of the associated molecules, a cellular tRNA, is tightly bound to the genome RNA and serves as the major primer for M-MuLV RNA-directed DNA synthesis in vitro.

RNA tumor virus particles such as murine leukemia virus or Rous sarcoma virus (RSV) contain two major size classes of RNA, the high-molecular-weight genome RNA and low-molecular-weight 4 to 7S RNA (1-3, 7, 8, 12, 19, 21, 25, 27). Some of the low-molecular-weight RNAs are associated with the genome RNA in a 70S complex, whereas others are released free in solution after disruption of virus particles (8, 12, 19, 21, 27). One of the 70S-associated 4S RNAs in RSV virions, the host cell $tRNA^{Trp}$, has been shown to be the primer for initiation of RSV RNA-directed DNA synthesis in vitro (6, 11, 14, 22).

To determine whether other viruses use tRNA's as primers, we analyzed the small RNAs of Moloney murine leukemia virus (M-MuLV). One of the 70S-associated 4S molecules can serve as the major primer for reverse transcription in vitro. This RNA dissociates from the genome RNA at higher temperatures than do other associated species. An identical molecule is found in uninfected mouse, rat, and chicken cells, suggesting that the M-MuLV primer is a host cell RNA. In a later communication, we will show that this molecule has the amino acid acceptor activity and nucleotide sequence of $tRNA^{Pro}$.

## MATERIALS AND METHODS

**Virus growth and isolation.** The cloned line of M-MuLV designated clone 1 was propagated in a 3T3 cell producer line according to published procedures (9, 10). For preparation of $^{32}P$-labeled viral RNA, infected cells were grown in phosphate-free Dulbecco medium containing 0.67 mCi of $^{32}PO_4^{3-}$ per ml and 10% dialyzed calf serum. After 10 h of labeling, unlabeled phosphate-free medium was applied to the cells for two 12-h periods. The three batches of medium were pooled, except in the preparation of long-term-labeled virus for which only the last harvest was used. Virus was purified by high-speed centrifugation and by sedimentation through sucrose gradients (9).

**Extraction and fractionation of viral RNA.** Viral RNA was extracted from $^{32}P$-labeled virions as described previously (21). The 70S RNA was separated from non-associated low-molecular-weight RNA by sedimentation through gradients of 5 to 20% sucrose containing 20 mM Tris-chloride (pH 7.8), 100 mM NaCl, 1 mM EDTA, and 0.5% Sarkosyl at 200,000 × g, for 1 h at 4°C in a Beckman SW50.1 rotor. In some experiments, the 70S RNA was dissociated by heating to 65°C for 2 min in a solution of 10 mM Tris-chloride (pH 7.5), 10 mM NaCl, and 1 mM EDTA. The high-molecular-weight subunits were separated from the released small RNA by centrifugation for 2 h in sucrose gradients, as described above.

The low-molecular-weight RNAs of M-MuLV were fractionated by two-dimensional polyacrylamide gel (2-D gel) electrophoresis and quantitated as described elsewhere (16, 21). RNA samples were applied to the gel in 1% agarose, to trap molecules too large to enter the gel, for subsequent quantitation. Total small RNAs were studied by heating viral RNA to 95°C for 2 min before application to the gel. Free small RNAs were analyzed in preparations that had not been heated above 40°C, to avoid dissociation of the 70S complex. Associated small RNAs were prepared from sucrose gradient-purified 70S RNA, which was then heated to 95°C before application to the gel.

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**Fingerprint analysis.** The separated RNA species, located in the gel by autoradiography, were eluted and digested with RNase T1. The resulting oligonucleotides were fingerprinted by the procedures of Sanger et al. (20). The fingerprints shown in this publication were obtained by scaling down the conventional method, so that four samples could be run on one piece (42 by 80 cm) of DEAE-cellulose paper. This was done by reducing the duration of electrophoresis in the first and second dimensions, respectively, to 75 min at 5 kV (80-cm cellulose acetate) and 250 min at 1.5 kV (80-cm DEAE-paper). Transfer from cellulose acetate to DEAE-paper was by the method of Southern (23), with the DEAE-paper attached to glass plates by double-sided tape (e.g., Scotch brand no. 665). The tape was subsequently removed by ethanol washing of the paper. Thin-layer chromatography on polyethyleneimine-cellulose was by the procedure of Griffin (13).

**Labeling of primer RNA.** Molecules in the 70*S* RNA complex which were capable of acting as primers for M-MuLV RNA-directed DNA synthesis were labeled in detergent-disrupted virions in which [$\alpha$-$^{32}$P]ATP was the only added deoxynucleoside triphosphate. Since the DNA synthesized on M-MuLV 70*S* RNA begins with -dApdA (15), this procedure radioactively tags the 3′ ends of any active primer molecules (5). Purified virus was suspended at 5 mg/ml in a 1-ml reaction mixture containing 50 mM Tris-chloride (pH 8.3), 50 mM NaCl, 6 mM $MgCl_2$, 5 mM dithiothreitol, 0.01% Nonidet P-40; 4 $\mu$M [$\alpha$-$^{32}$P]dATP (New England Nuclear Corp.; ~100 Ci/mmol), 30 mM creatine phosphate, and 0.01 mg of creatine phosphokinase per ml. In some experiments, $^{32}$P-labeled virions were used to direct DNA synthesis, with 40 $\mu$M [$^3$H]dATP (New England Nuclear Corp.; 20 Ci/mmol) as the only added nucleoside triphosphate.

After incubation at 37°C for 30 min, the reaction was terminated by adding a 5-ml solution of 10 mM Tris-chloride (pH 7.5), 10 mM EDTA, and 200 mM sodium acetate and extracting twice with a phenol-chloroform mixture (1:1). The phenol-extracted products were either denatured and analyzed directly by 2-D gel electrophoresis or fractionated on sucrose gradients as described above, prior to denaturation and gel electrophoresis.

## RESULTS

**Small RNAs of M-MuLV: (i) Polyacrylamide gel analysis.** The low-molecular-weight RNAs of $^{32}$P-labeled M-MuLV virions were fractionated by 2-D gel electrophoresis (Fig. 1). Figure 1a is the pattern obtained for total small RNAs. As illustrated schematically in Fig. 1b, about 15 fractions of 4*S* RNA can be identified. A numbering system for these RNAs, not related to that used for RSV RNAs, is presented. In addition, two conformational isomers of 5*S* RNA and three species designated 5.5*S*, 6*S*, and 7*S* (based on relative electrophoretic mobilities) can be seen.

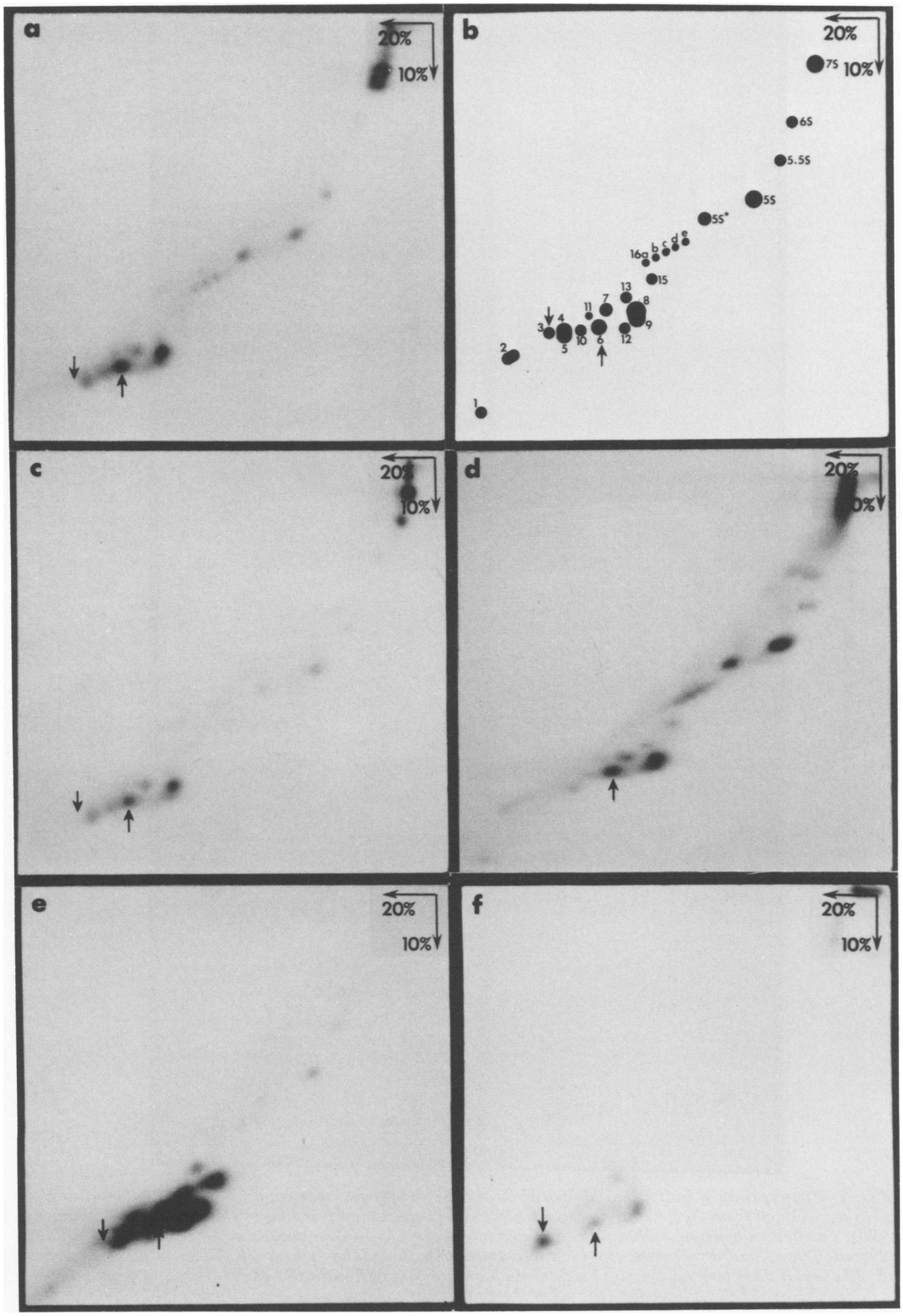
The patterns of free and associated small RNAs are shown in Fig. 1c and d, respectively. Although the relative intensities of spots in Fig. 1c and d differ, the patterns of spots are similar. By fingerprint analysis, discussed below, similarly migrating RNAs in the two preparations were shown to be identical. The major qualitative difference between the free and associated preparations is the presence of species 16a through e exclusively in the associated RNA.

The pattern of small RNAs in M-MuLV is relatively simple when compared with that of 4 to 5*S* RNA from the host 3T3 mouse cells (cf. Fig. 1a and e). This difference in complexity is consistent with the M-MuLV 4*S* RNAs being a subset of the cellular tRNA population. Also included for comparison is the free 4*S* RNA from the Schmidt-Ruppin-D strain of RSV (Fig. 1f). The patterns observed for M-MuLV and RSV are clearly different, although some of the RNAs, for instance those indicated by the arrows in Fig. 1a through f, are found in both viruses, as well as in uninfected chicken embryo fibroblast, mouse (3T3) and rat (NRK) cells (as determined by fingerprint analysis in all cases).

**(ii) Fingerprints of individual RNAs.** All of the RNAs corresponding to the numbered spots in Fig. 1b were eluted and characterized by RNase T1 digestion and oligonucleotide fingerprinting. Figure 2 shows some representative fingerprints. Many of the oligonucleotide patterns are relatively simple, with a complexity usually found for molecules of tRNA size. Other spots yielded too many oligonucleotides to have been derived from a single 4*S* RNA and, therefore, probably contained two or more species.

Fingerprints of RNAs in spots 1, 4, 7, 8, 13, and 15 were sufficiently simple to indicate that these RNAs were quite pure after electrophoresis. RNAs in spots 2, 10, 11, and 12 were mixtures of two or more molecules. Spot 5 from free RNA was always contaminated by spot 4 RNA.

FIG. 1. *2-D gel electrophoresis patterns of $^{32}$P-labeled small RNAs from M-MuLV, 3T3 cells, and RSV. The first dimension was in 10% acrylamide (top to bottom), and the second dimension was in 20% acrylamide (right to left) as described in the text. (a) Total M-MuLV small RNAs; (b) schematic drawing of total M-MuLV small-RNA pattern showing numbering system used; (c) free M-MuLV small RNAs; (d) 70S RNA-associated M-MuLV small RNAs; (e) 4 to 5S RNA from 3T3 cells; (f) free small RNAs from RSV. Upward arrows indicate the position of a major M-MuLV tRNA common to all the patterns shown. Downward arrows indicate the position of tRNA$^{Trp}$, the primer for RSV RNA-directed DNA synthesis in vitro.*


a
20%
10%
b
20%
10%
75
6S
5.5S
5S
5S*
16a
b
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13
11
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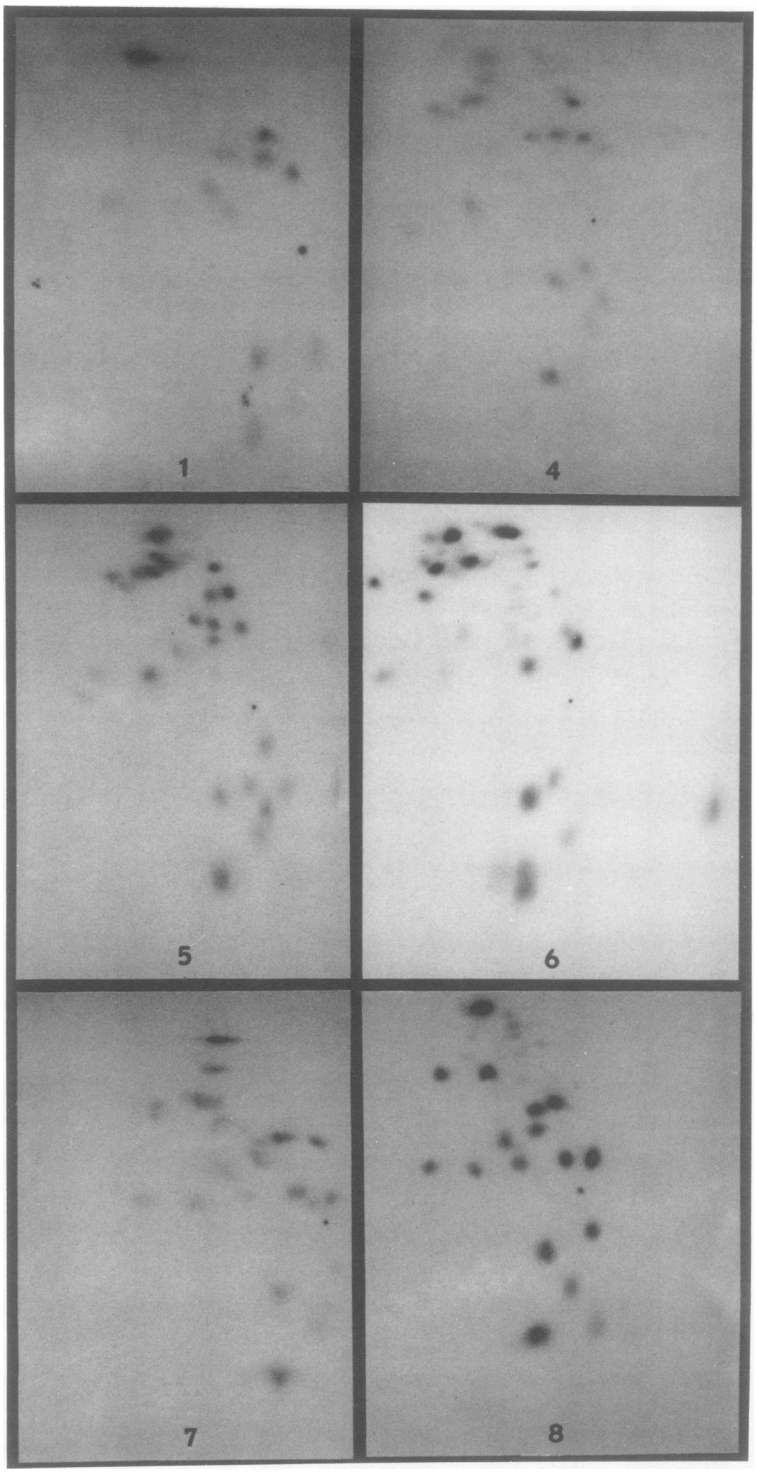


FIG. 2. *Fingerprints of individual M-MuLV RNAs. RNA species corresponding to the numbered spots in Fig. 1b were eluted from the gel and digested with RNase T1 for fingerprinting by the procedures of Sanger et al. (20). The first dimension, right to left, was electrophoresis on cellulose acetate in pyridine acetate (pH 3.5)-6 M urea. The second dimension, from top to bottom, was electrophoresis on DEAE-cellulose in 7% formic acid. The small dark spot on the right side of each autoradiograph indicates the position of the blue marker dye (xylene-Cyanol FF). Except for 16c, all of the fingerprints shown were derived from free RNAs. A schematic representation of the oligonucleotide pattern from the associated 16a through e series is included.*

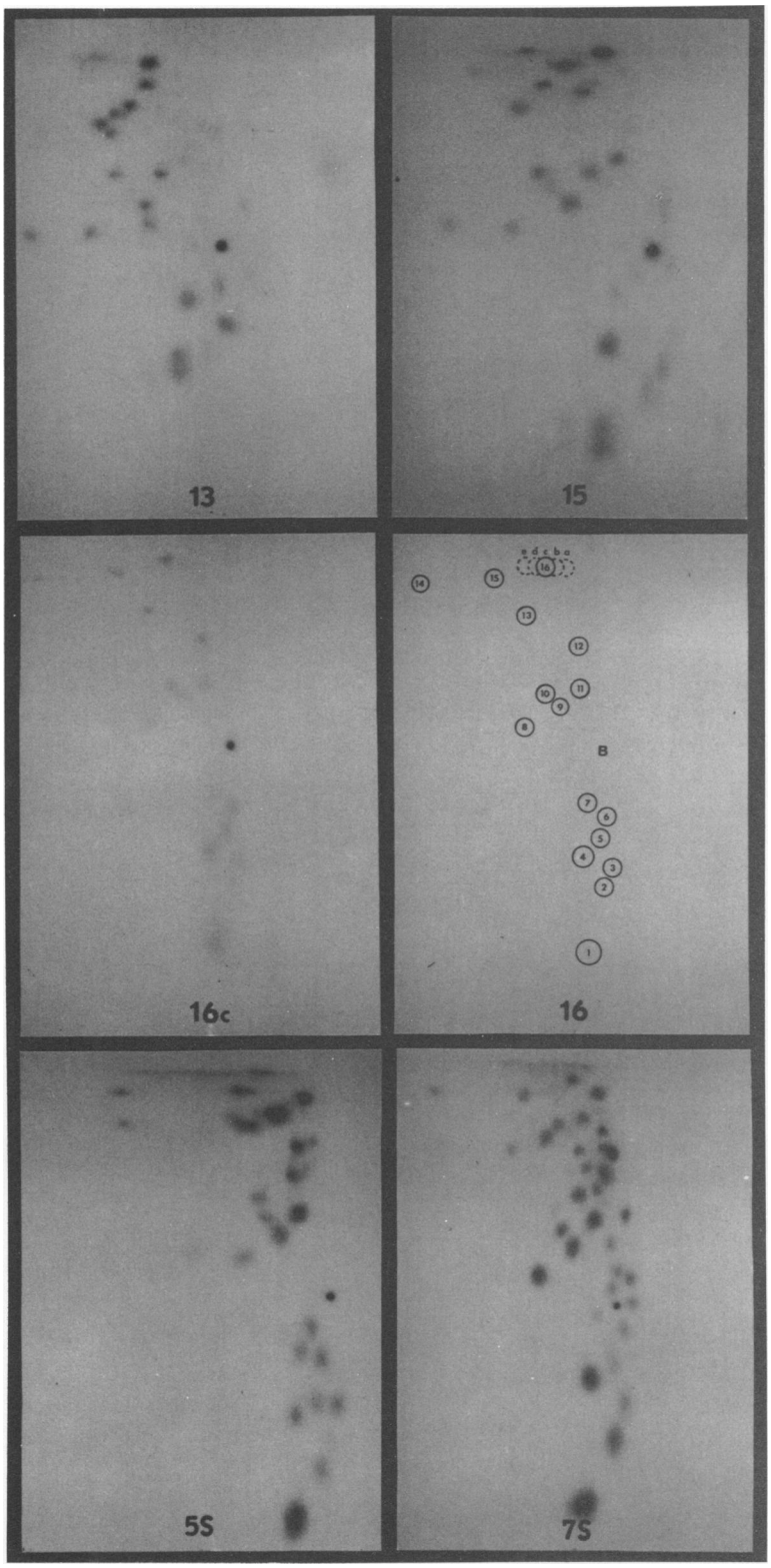


*Fig. 2 cont.*

However, when purified from associated small RNAs, spot 5 was uncontaminated, showing that spot 4 RNA was free but probably not associated. The fingerprint of the RNA in spot 8 was very similar to that of spot 7, indicating that this molecule migrated in at least two positions in the gel. This same fingerprint was also obtained from RNAs of RSV (called RSV spot 5 by Sawyer and Dahlberg [21]). Likewise, the oligonucleotides of M-MuLV spot 4 are a subset of those found for RSV spot 1b; in both viruses, this RNA is only in the free fraction.

The RNA in spot 6, a major component in both the free and associated fractions, was primarily a single species, but there appeared to be about 25% contamination by another molecule.

If the major RSV small RNA, $tRNA^{Trp}$, were present in virions of M-MuLV, it should have the 2-D gel mobility of spot 3. The RNase T1 oligonucleotides characteristic of $tRNA^{Trp}$ could not be identified in the fingerprint of spot 3, presumably because another RNA predominated in that spot. However, the presence of $tRNA^{Trp}$ in M-MuLV RNA was established by fingerprint analysis of a molecule from M-MuLV 4*S* RNA which formed a complex with avian myeloblastosis virus DNA polymerase (17).

In M-MuLV RNA, several spots (denoted 16a through e in Fig. 1b) form a line on the gel. Fingerprints of the RNAs in these spots are identical, except for one oligonucleotide near the top of the schematic drawing shown in Fig. 2-16. The change in mobility of this oligonucleotide is consistent with the larger RNAs having progressively more uridylic acid residues at one end. Comparison of Fig. 1c and d shows that this series of RNAs is in the associated, but not in the free, fraction. The small amount of material in the free sample migrating in the position of 16a had a fingerprint quite unrelated to that of associated spot 16a RNA.

**(iii) Quantitation of small RNAs.** Figure 1 shows that the small RNAs of M-MuLV were not present in equal amounts. The average number of molecules of each RNA in the free and associated fractions, relative to one 35*S* RNA subunit, were calculated assuming that the genomic and small RNAs were uniformly labeled by $^{32}PO_4^{3-}$ (21). The gel pieces corresponding to each of the numbered spots in Fig. 1b were cut out, and the amount of $^{32}P$ was determined by counting Cerenkov radiation prior to elution and fingerprint analysis. Quantitation was made relative to the high-molecular-weight RNA trapped in the agarose at the gel origin as described in Materials and Methods. The high-molecular-weight RNA was assumed to be all 35*S* with a chain length of 10,000 nucleotides, whereas the 4*S*, 5*S*, and 7*S* RNA molecules were assumed to be 75, 120, and 240 nucleotides, respectively. Other chain lengths were computed from the logarithmic relationship between electrophoretic mobility and molecular weight in the 10% gel (18).

Table 1 (long-term label) contains quantitation of the average copy number of each small RNA per 35*S* subunit. Many of the small RNAs appeared to be present in less than one copy per 35*S* subunit. In contrast to RSV, there was not a single major 4*S* species; spots 6, 8, and 9, present in virtually equivalent amounts, predominated in both the free and, more strikingly, the associated fraction. Approximately three to four copies of spot 6 RNA per 35*S* subunit were free, and one-half to one copy was associated. The proportion of 5*S* RNA relative to other small RNAs was increased in the associated fraction, with roughly 0.3 to 0.5 molecules per 35*S* subunit in the 70*S* RNA complex. These quantitations were subject to considerable error, and repeat experiments agreed only to within ±25% of the values presented in Table 1.

When the virus was labeled for shorter times, the amounts of $^{32}P$ in most of the small RNAs decreased relative to the label in the genomic RNA (Table 1). This result suggested that the two populations of virion RNAs, small RNAs and genomic RNA, were derived from two different intracellular pools. Such a difference would be expected if the virion tRNAs were taken up from the large pools of mature cell tRNAs, which would be slow to attain constant specific activity (6). Members of the 16a–e series were again exceptional in that the amount relative to genomic RNA was unaffected by the length of the labeling protocol.

**(iv) Stability of 4*S* RNA-genomic RNA complexes.** To determine whether 35*S* genome RNA binds particular small RNAs more tightly than others, the high-molecular-weight RNA complex was heat denatured in two steps. Small RNAs released below 65°C were compared with those that remained bound at 65°C but were released at 95°C (Fig. 3a and b). Most of the associated small RNAs, including 16a through e and 5*S* RNA, were released below 65°C (cf. Fig. 3a with Fig. 1d). In contrast, one small RNA remained associated at 65°C and was released by heating to 95°C. The gel mobility and RNase T1 fingerprint of this tightly associated RNA showed that it was the RNA of spot 6. In some experiments, a minor species of RNA, with a mobility similar to that of spot 5

TABLE 1. *Quantitation of M-MuLV small RNAs relative to 35S genomic RNA*[a]

| Spot | Assumed no. of nucleotides | Long-term label | | Short-term label | |
|---|---|---|---|---|---|
| | | Associated copies | Free Copies | Associated copies | Free copies |
| 1 | 50 | 0.11 | 0.44 | 0.04 | ND |
| 2 | 60 | 0.17 | 0.36 | 0.07 | ND |
| 3 | 75 | | 0.42 | | 0.08 |
| 4 | 75 | | 1.35 | | 0.08 |
| 5 | 75 | 0.11 | 2.20 | 0.04 | 0.32 |
| 6 | 75 | 0.87 | 4.03 | 0.20 | 1.12 |
| 7 | 75 | 0.13 | 1.58 | 0.11 | 0.49 |
| 8 | 75 | 0.24 | 3.96 | 0.20 | 1.57 |
| 9 | 75 | 0.25 | 4.47 | 0.22 | 0.60 |
| 10 | 75 | 0.08 | 2.27 | 0.04 | 0.41 |
| 12 | 75 | 0.17 | 0.94 | 0.10 | 0.28 |
| 13 | 75 | 0.07 | 0.72 | 0.05 | 0.17 |
| 15 | 75 | 0.13 | 0.40 | 0.07 | 0.14 |
| 16a | 100 | 0.04 | | 0.04 | |
| 16b | 100 | 0.07 | | 0.05 | |
| 16c | 100 | 0.09 | | 0.08 | |
| 16d | 100 | 0.09 | | 0.08 | |
| 16e | 100 | 0.05 | | 0.08 | |
| 5*S* | 120 | 0.32 | 0.43 | 0.43 | 0.18 |
| 5.5*S* | 150 | 0.05 | 0.25 | 0.04 | 0.05 |
| 6*S* | 200 | 0.02 | 0.11 | 0.06 | 0.04 |
| 7*S* | 240 | 0.31 | 0.51 | 0.40 | 0.40 |
| 35*S* | 10,000 | 1.0 | 1.0 | 1.0 | 1.0 |

[a] The pieces of gel corresponding to the spots numbered in Fig. 1 were cut out, and the radioactivity was measured by Cerenkov counting. For reference, the high-molecular-weight genomic RNA trapped in agarose at the origin of the 10% gel (see Materials and Methods) was also counted (21). The high-molecular-weight RNA was assumed to be all 35*S* with a chain length of 10,000 nucleotides, whereas the 7*S*, 5*S*, and 4*S* RNAs were assumed to be 240, 120, and 75 nucleotides, respectively. Other chain lengths were computed assuming a logarithmic relationship between molecular weight and electrophoretic mobility in the 10% gel. The number of copies of each RNA was calculated by dividing the $^{32}P$ counts per minute by the number of nucleotides (phosphates) in the molecule and normalizing the data to 1.0 copy of 35*S* RNA. It was assumed that the small RNAs and genomic RNAs were labeled to the same specific activity. As discussed in the text, this assumption probably holds for long-term labeling, in which the virions were harvested 36 h after labeling, but not for the short-term labeling.

but with a different fingerprint, was also released at elevated temperatures.

**Identification of the primer RNA for M-MuLV DNA synthesis: (i) Tagging with [α-$^{32}P$]dATP.** Synthesis of RNA tumor virus DNA is initiated by extension of an RNA primer (4, 26). The primer can be radioactively tagged by the addition of a few deoxynucleotides in a limited DNA synthesis reaction, using an unlabeled template-primer complex and radioactive deoxynucleoside triphosphate precursors (6). Since the initial sequence of M-MuLV DNA is -dApdApdT (15), the M-MuLV primer RNA was labeled by including [α-$^{32}P$]dATP as the only precursor in DNA synthesis reactions, using either detergent-disrupted virions or 70*S* RNA plus M-MuLV reverse transcriptase.

After synthesis, the primer RNA, elongated by one or two dAMP residues, was dissociated from the template by heating to 95°C and was analyzed by 2-D gel electrophoresis. Most of the dAMP-labeled primer migrated as a single spot in the gel (Fig. 3c). The mobility of the RNA in this spot corresponded to that of spot 6 RNA, as determined by re-electrophoresis of a mixture of the two molecules (Fig. 3d). This result suggested that spot 6 RNA served as the primer for M-MuLV DNA synthesis.

**(ii) Fingerprinting the primer.** Although the tagged primer and spot 6 RNA co-migrated, it was possible that the primer RNA had shifted to the position of spot 6 as a result of dAMP addition to the 3′ end. If that were the case, we would expect to find a new set of oligonucleotides in the fingerprint of spot 6 RNA after the tagging reaction. This possibility was tested in a double-label experiment in which template-primer, uniformly labeled with $^{32}P$, was tagged with [$^{3}H$]dATP, and the small RNAs were purified by 2-D gel electrophoresis. The material in spot 6 was eluted and fingerprinted. Before digestion with RNase T1, a small amount of [α-

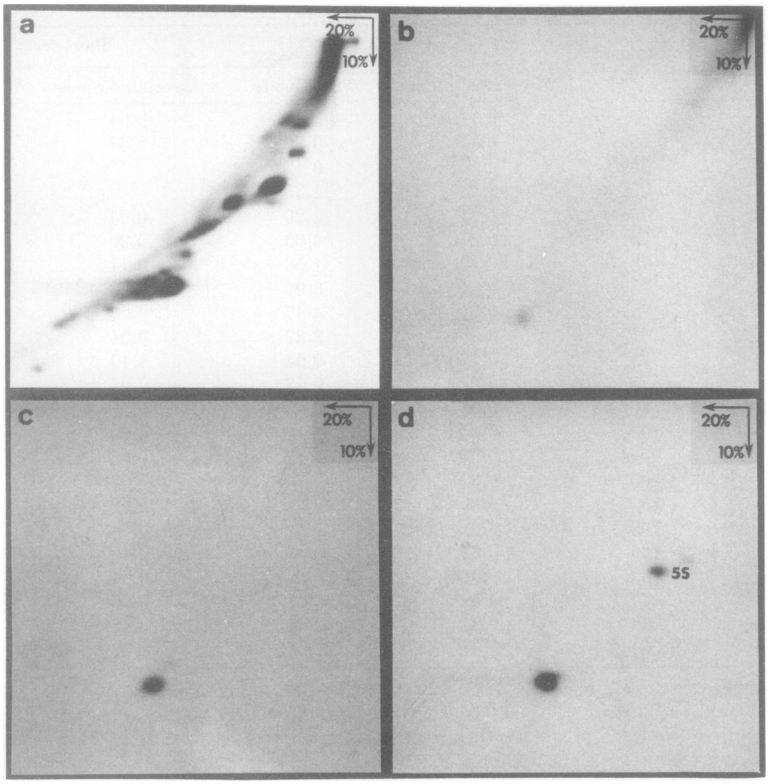


FIG. 3. *Identification of a primer RNA for M-MuLV DNA synthesis. Uniformly $^{32}$P-labeled M-MuLV 70S RNA was heated sequentially to 65°C and to 95°C in a solution of 10 mM Tris-chloride (pH 7.5), 10 mM NaCl, 1 mM EDTA as described in the text. The small RNAs released below 65°C (a) and between 65 and 95°C (b) were analyzed by 2-D gel electrophoresis. (c) Unlabeled M-MuLV virions were used to direct DNA synthesis in a reaction containing [α-$^{32}$P]dATP as the only deoxynucleoside triphosphate. The products were phenol extracted and analyzed by 2-D gel electrophoresis. Small RNAs tagged with [$^{32}$P]dAMP were detected by autoradiography. (d) Co-electrophoresis of [$^{32}$P]dAMP-tagged primer and uniformly $^{32}$P-labeled M-MuLV spot 6 RNA in 2-D gels. Equal amounts ($^{32}$P counts per minute) of the two samples were applied to the gel along with 5S RNA as a marker.*

$^{32}$P]dATP-tagged primer was added to aid in location of the $^{3}$H-tagged end. The fingerprint obtained (Fig. 4) was very similar to that of pure spot 6 RNA (Fig. 2-6), except for the addition of two new oligonucleotides (5a and 5b) near the position of the normal 3′-terminal oligonucleotide, no. 5 (Fig. 4). These new spots on the fingerprint were the only ones that contained $^{3}$H as assayed by scintillation counting of regions of the fingerprint containing $^{32}$P. Since the only new oligonucleotides to appear in the fingerprint of the RNA in spot 6 after DNA synthesis were those to which the nascent DNA was attached, we concluded that the nascent DNA was added to spot 6 RNA.

In the [α-$^{32}$P]dATP tagging experiment illustrated in Fig. 3c, about 10% of the tagged material migrated more slowly than did spot 6 RNA. No specific RNA molecule with this mobility was detected in uniformly $^{32}$P-labeled viral RNA regardless of whether or not DNA synthesis had been carried out, although there was always background in that region of the gel. To improve the resolution of this material on the gel, uniformly $^{32}$P-labeled viral RNA tagged with $^{3}$H was subjected to differential heat treatment as described above. In this case, only RNAs that dissociated from the template between 65 and 80°C were analyzed. The four $^{32}$P-labeled fractions of RNA that were observed after 2-D gel electrophoresis (Fig. 5) were eluted and fingerprinted. The fingerprints of RNAs in spots b, c, and d were identical to each other and to spot 6, except for oligonucleotides that corresponded to the 3′ ends (Fig. 5). The fingerprints of the RNAs in spots b and c differed only in the relative amounts of untagged and singly or doubly $^{3}$H-tagged 3′-terminal oli-

gonucleotides. Spot d RNA also had the fingerprint of spot 6 RNA, except that we were unable to locate the 3′ end or an oligonucleotide on the fingerprint containing the $^3H$ tag. Since radioactive material in the position of spot d was observed only after DNA synthesis, we feel that it represents primer on which the nascent DNA was elongated by several additional nucleotides. The RNA denoted as spot "a" contained no detectable $^3H$ and could correspond to the minor species that was occasionally released above 65°C (see above).

## DISCUSSION

About 30% of the RNA in virions of M-MuLV is of low molecular weight, between 4 and 7*S*. We have purified many of these molecules and characterized them by their electrophoretic mobilities in 2-D gels, by their oligonucleotide fingerprints, and by whether they are found free or associated with the high-molecular-weight genomic RNA. One of the 4*S* RNA species was shown to act as the primer for the virion-associated, RNA-directed DNA polymerase.

After disruption of M-MuLV virions, most of the small RNA is free, but a specific subset is associated with the genomic RNA. This association does not necessarily reflect the state of the RNAs within the virus particle, since the RNAs were prepared by phenol extraction, which could denature some complexes (24). Statements about the presence or absence of a particular RNA in preparations of viral RNAs depend on there being sufficient radioactivity for it to appear as a spot in the autoradiograph. As a result, the data presented here may be biased in favor of the RNAs that are present in relatively high amounts and are well resolved from other RNAs on the gel.

Among the associated small RNAs, the primer RNA was identified by its ability to accept deoxynucleotides in a DNA synthesis reaction. The 2-D gel mobility and the fingerprint of the M-MuLV primer RNA showed that

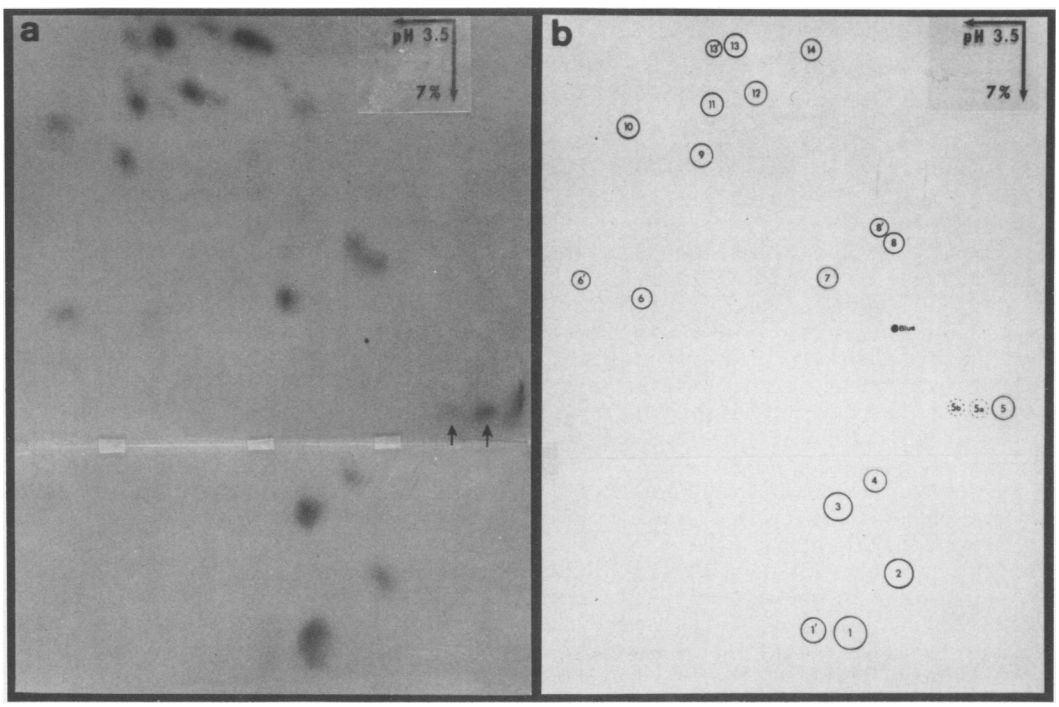


FIG. 4. *Fingerprint of M-MuLV spot 6 RNA after limited DNA synthesis. Uniformly $^{32}P$-labeled M-MuLV virions were used to direct DNA synthesis in a reaction containing [$^3H$]dATP as the only deoxynucleoside triphosphate. Spot 6 RNA was purified from the reaction mixture by 2-D gel electrophoresis and eluted for fingerprinting. Prior to RNase T1 digestion, a small amount of [$^{32}P$]dAMP-tagged primer RNA was added to aid in the identification of any oligonucleotides tagged during DNA synthesis. Two additional oligonucleotides are apparent in the fingerprint of spot 6 RNA, denoted by arrows in (a) and numbered 5a and 5b in the schematic diagram (b). Oligonucleotide 5 is the normal 3′-terminal oligonucleotide of spot 6 RNA (Harada, Peters and Dahlberg, in preparation).*

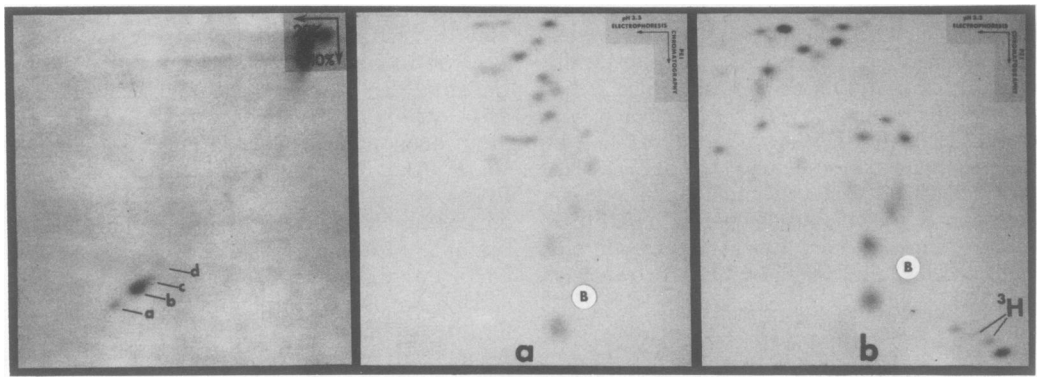


FIG. 5. *2-D gel electrophoresis and fingerprinting of material tightly bound to genomic RNA after limited DNA synthesis. Uniformly $^{32}$P-labeled M-MuLV virions were used to direct DNA synthesis in a reaction containing [$^3$H]dATP as the only deoxynucleoside triphosphate. The 70S RNA complex was isolated from the reaction mixture and subjected to differential melting as described in the legend to Fig. 3. The left panel shows the result of 2-D gel electrophoresis of material remaining associated with 35S genomic RNA at 65°C but released at 95°C. The mobility of spot b corresponds to that of M-MuLV spot 6, whereas molecules with the mobilities of spots a, c, and d are not normally detected in uniformly labeled viral RNA. The middle and right panels are the RNase T1 fingerprints of the RNAs in spots denoted a and b, respectively. The second dimensions of the fingerprints were carried out by thin-layer chromatography on polyethyleneimine plates as described by Griffin (13). B denotes the position of the blue dye marker (xylene-Cyanol FF). $^3$H indicates the position of those oligonucleotides containing tritium.*

it was the major RNA of spot 6. As in the case of RSV (6, 21), the M-MuLV primer RNA was the most abundant (about one copy per 35*S* RNA subunit) and the most stably bound associated small RNA.

The source of the low level of contaminating oligonucleotides seen in the fingerprints of most preparations of spot 6 RNA is unclear. We note that many of the minor oligonucleotides are also found in spots c and d of Fig. 5, in which the mobility of the primer was altered slightly by the addition of deoxynucleotides. It is unresolved, therefore, whether the minor oligonucleotides result from other (perhaps modified) forms of spot 6 RNA, a low level of background RNA, or a second primer with the same gel mobility as the major primer RNA.

In experiments to be reported elsewhere, we have shown that the major RNA of spot 6 is $tRNA^{Pro}$ (F. Harada, G. Peters, and J. E. Dahlberg, manuscript in preparation). That work includes aminoacylation of the primer as well as determination of the entire nucleotide sequence. Waters has shown that in virions of AKR virus, the small RNA most stably bound to the genomic RNA is $tRNA^{Pro}$ (28).

The results presented here for the small RNAs of M-MuLV are comparable to those obtained previously in our analysis of the small RNAs of RSV (5, 6, 11, 21, 22). Although the results obtained for RSV and M-MuLV are analogous, the molecules involved are different in the two systems. Some small RNAs are present in virions of both RSV and M-MuLV, e.g., the molecules in spots 4, 6, and 8, 5*S* and 7*S* RNAs (Fig. 1). In contrast, several RNAs are present in only one of the viruses. The RNAs of spots 1, 13, and 15 were not identified in RSV virions (21), whereas $tRNA^{Trp}$ and $tRNA_4^{Met}$, which are major RSV RNAs (called spots 1 and 6, respectively, in reference 21), are barely detectable among M-MuLV RNAs.

As is the case with other RNA tumor viruses, the small RNAs of M-MuLV appear to be normal components of the host cell (1, 2, 22, 27). We have isolated, fingerprinted, and sequenced the major RNA of spot 6 from uninfected chicken embryo fibroblasts, mouse (3T3), and rat (NRK) cells grown in tissue culture. This RNA, $tRNA^{Pro}$, appears to have the same structure in all these cells. The kinetics of labeling the M-MuLV virion small RNAs and the genomic RNA indicate that the low-molecular-weight RNAs are derived from a large intracellular pool. Many of the M-MuLV small RNAs contain modified nucleotides characteristic of tRNAs.

The RNA in the series 16a through e is unusual in that it is found only in the associated fraction. It is displaced from the 70*S* RNA complex by heating to 65°C (Fig. 3a). The RNAs in spots 16a through e are structurally related, as determined by their RNase T1 fingerprints, which differ in only one oligonucleotide. Preliminary experiments show that they do not contain modified nucleotides characteristic of

tRNA. Since the apparent amount of 16a through e relative to the high-molecular-weight RNA does not vary with the length of time that infected cells are labeled (Table 1), we conclude that this molecule(s) does not pass through a large intracellular pool. It is possible that it is a degradation product of the genomic RNA. Alternatively, it may have a specific function, such as holding the 35S RNA subunits together in the 70S complex.

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