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Comparison of the Small RNAs of Polymerase-Deficient and Polymerase-Positive Rous Sarcoma Virus and Another Species of Avian Retrovirus

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The small RNAs contained in virions of avian leukosis and sarcoma viruses are a virus-specific subset of the total small RNA population of the host cell. The reverse transcriptase protein must be present in the budding virion for this selection to take place. Virions of the α form of the Bryan strain of Rous sarcoma virus, which lack detectable reverse transcriptase, incorporated an unselected population of small RNAs identical to total chicken cell small RNA. Virions of reticuloendotheliosis virus, which contain a reverse transcriptase unrelated to that of the avian leukosis and sarcoma viruses, contained a distinctly different population of small RNAs although both the avian leukosis and sarcoma and the reticuloendotheliosis viruses were grown in chicken cells. Because the primer for avian leukosis and sarcoma virus RNA-dependent DNA synthesis is a host cell tRNA, the differences in reverse transcriptase small RNA selection may help explain the failure of different species of retrovirus to complement for the reverse transcriptase.

Virions of Rous sarcoma virus (RSV) contain two major size classes of RNA: a 70S genomic RNA and a mixture of 4 to 7S RNA species. The majority of the small-sized RNAs in the virion are not associated with the 70S complex when RNA is extracted from virions with sodium dodecyl sulfate alone or with phenol (1-3, 7, 8, 12, 37). The 70S RNA itself is a complex of two identical 35S subunits that can be dissociated upon heating. Heat denaturation also releases small amounts of 4 to 7S RNAs from the 70S complex (10, 14, 37). One of the 70S-associated small RNAs has been identified as the major primer for in vitro RNA-dependent DNA synthesis catalyzed by the viral reverse transcriptase (6, 13, 15). The virion small RNAs can be separated into about 10 discrete species by two-dimensional (2-D) polyacrylamide gel electrophoresis (23, 37). Nucleotide sequence analysis of individual species of 4 to 7S RNA isolated in this manner has shown them to be identical to molecules found in normal chicken cells and led to the conclusion that both the 70S-associated and free virion small RNAs are of host rather than viral origin (11, 21, 38). One of these RNAs acts as the major primer in vitro for the reverse transcriptase of avian leukosis and sarcoma viruses (ALSV) and has been identified as a cellular tryptophanyl tRNA ($tRNA_{TRP}$) (21). The small RNAs present in virions, however, are not a random population of host cell small RNAs. Rather, the major species of small RNA packaged into RSV virions are a very specific subset of the total host small RNAs (39). However, little is known about the mechanism by which only certain host small RNA species are packaged into virions.

Two observations prompted us to examine the role of the polymerase in the selection of small RNAs for packaging. First, Panet et al. demonstrated that when purified avian myeloblastosis virus polymerase was mixed with total chicken cell tRNA only $tRNA_{TRP}$ was bound in substantial amounts (31). Second, we had observed that virions of the α form of the Bryan strain of RSV (BH-RSVα), a polymerase mutant apparently lacking any polymerase protein (17, 18, 35), seemed to contain less 4 to 7S RNA in relation to the 60 to 70S RNA than did the polymerase-positive BH-RSV (unpublished data).

We shall present evidence in this paper that the ALSV polymerase molecule is primarily responsible for determining which small RNAs are to be packaged and that a different species of retrovirus when grown on the same cell type selects different small RNAs.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo cells used in these studies were obtained from fertile eggs (SPAFAS, Inc., Norwich, Conn.). All primary cultures were

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tested for group-specific (gs) antigen and chicken helper factor (chf) expression and were of the C/E phenotype (19, 20). Chicken cells (gs^+chf^+) transformed by BH-RSV(−) were prepared by infection in the presence of UV-treated Sendai virus as described by Hanafusa et al. (19, 25). Cultures of BH-RSVα (polymerase negative)-transformed gs^+chf^+ chicken cells were grown from individual soft-agar colonies as follows. Secondary cultures of chicken embryo cells (7×10^6 cells in a 100-mm plate) were inoculated with 100, 200, or 400 focus-forming units of BH-RSVα-Rous-associated virus type 1 (RAV-1). After a 4-h adsorption, the cells were trypsinized, divided into six plates, and cultured in soft agar medium containing antiserum against RAV-1 to prevent the spread of the helper virus. After 12 to 17 days, individual colonies were picked from plates containing less than 50 colonies and incubated for 10 to 15 min at room temperature in 0.1 ml of trypsin-EDTA solution (0.05% trypsin-0.02% EDTA in Tris-saline buffer: 0.025 M Tris-hydrochloride [pH 7.4]-0.1 M NaCl-0.005 M KCl-0.003 M NaH_2PO_4-0.006 M glucose). The cells were then plated on 35-mm plates containing 4×10^5 normal (gs^+chf^+) chick cells. Cultures were kept under soft agar medium and transferred periodically until they contained primarily transformed cells. BH-RSVα-transformed cultures were identified by a lack of infectious transforming virus and virus-associated RNA-dependent DNA polymerase activity in the culture fluid and by positive rescue of infectious sarcoma virus after superinfection with RAV-1 (15, 16). The media, conditions for tissue culture, and preparation of RAV-1, RAV-2, and BH-RSVα(RAV-1) pseudotypes have been described previously (20, 25). RAV-1 and RAV-2 were grown on gs^-chf^- chicken cells. BH-RSVα is routinely grown in gs^+chf^+ chick cells to enable us to test for low levels of BH-RSV(−) polymerase-positive contamination by focus assay on quail cells. The focus assay is much more sensitive than the polymerase assay. Spleen necrosis virus and reticuloendotheliosis virus (strain T) (REV-T) were kindly supplied by H. Temin, University of Wisconsin, Madison, and were grown on gs^-chf^- chick cells. Spleen necrosis virus and REV-T stocks were determined to be free from contaminating leukosis virus by testing for interference with BH-RSV pseudotypes of various subgroups.

Polymerase assay. Virus-associated RNA-dependent DNA polymerase activity was determined with virus pellets obtained by centrifuging 10 ml of culture fluid in a Beckman type 40 rotor. The reaction mixture in 0.1 ml contained: 7.5 μg of $(rC)_n \cdot (dG)_{12-18}$ (7:3), 0.1% Nonidet P-40, 5.0 μmol of Tris-hydrochloride (pH 8.3), 2.0 μmol of NaCl, 1.0 μmol of magesium acetate (for ALSV) or 0.2 μmol of manganese chloride (for REV), and 500 pmol of [^{3}H]dGTP (500 cpm/pmol). The reactions were incubated at 37°C for 60 min, and then trichloracetic acid-precipitable radioactivity was determined.

Preparation of ^{32}P-labeled RNA. Cultures of the Schmidt-Ruppin strain of RSV (SR-RSV)-, BH-RSV-, and BH-RSVα-transformed chicken cells and RAV-2, REV-T-infected chicken cells were grown in 100-mm plastic culture plates. For $^{32}PO_4^{3-}$ labeling the regular culture medium was replaced with phosphate-free modified Eagle minimal essential medium containing 1 mCi of $^{32}PO_4^{3-}$ per plate and supplemented with 2% calf serum or 2% fetal calf serum. After 48 h of phosphate labeling the $^{32}PO_4^{3-}$-containing medium was replaced, and labeling was continued for an additional 24 h. In some experiments the cells were first grown in PO_4^{3-}-free medium for 24 h prior to the addition of ^{32}P. In these experiments the first harvest of ^{32}P-labeled virus was done after 24 h of labeling and at 24-h intervals thereafter. RNA was prepared immediately from each harvest individually without pooling, as previously described (37). Briefly, the purification procedure included a low-speed spin to remove cellular debris, a high-speed spin to pellet virus, banding of the virus in a linear sucrose gradient (10 to 65%), treatment with Pronase and sodium dodecyl sulfate, phenol extraction, chloroform-isoamyl alcohol extraction, and ethanol precipitation. In some experiments with virions other than BH-RSVα, the sucrose gradient step was eliminated and RNA was prepared directly from pelleted virions. We could detect no difference in the small RNAs prepared by either procedure.

70S virion RNA and free small RNAs were prepared by rate zonal centrifugation in 5-ml or 12-ml 5 to 20% sucrose gradients in TSE buffer in a Beckman SW50.1 or SW40 rotor, respectively. (TSE = 0.02 M Tris-hydrochloride [pH 7.2]-0.1 M NaCl-0.001 M EDTA).

2-D polyacrylamide gel electrophoresis. The virion or cellular small RNAs were separated from each other by a 2-D polyacrylamide gel electrophoresis technique (23, 37). All gels were run in an EC-470 or EC-490 cell (E-C Corp., St. Petersburg, Fla.). The first dimension was subjected to electrophoresis 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 0.5× dilution of the TEB buffer of Peacock and Dingman (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 or 17 by 1 cm, containing 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 the first dimension strip. After polymerization the gel was subjected to electrophoresis for 18 h at 400 V and 15°C with 0.5× TEB buffer.

Samples were loaded on the gel in a mixture of buffer and hot (65°C) 2% agarose which then solidified in the sample slot. Since only RNA molecules about 300 nucleotides long or smaller entered the 10% polyacrylamide gel, the larger RNAs were trapped at the origin by the agarose for quantitation.

After electrophoresis, gels were sealed in plastic, and the small RNA patterns were visualized by autoradiography using Cronex II X-ray film (DuPont).

Quantitation of small RNAs. Individual small RNAs were located in the 2-D gel by aligning the autoradiograpm over the gel. The area of the gel containing the RNA was cut out, and the amount of radioactivity was determined in a liquid scintillation counter. Similarly, the amount of radioactivity (in counts per minute [cpm]) in high-molecular-weight

(HMW) RNA was determined from the material remaining in the sample slot. The number of copies of a small RNA relative to 70S RNA was calculated from the formula: number of copies = cpm in spot X/[cpm in HMW RNA × (number of nucleotides in spot X/20,000)]. In calculating numbers of copies per virion, each virion is assumed to contain one 70S RNA of 20,000 nucleotides. In a previous publication (37), this quantitation was done assuming a 70S RNA of 30,000 nucleotides, which would result in larger estimates of copy numbers.

RESULTS

To examine the role of the polymerase molecule in the selection of small RNAs we prepared cultures of gs^+chf^+ chicken cells transformed by BH-RSV (polymerase positive) and BH-RSVα (polymerase negative). The latter cultures release noninfectious particles lacking the viral polymerase molecule (17, 18, 35). The cultures were labeled with ^{32}P in phosphate-free medium, and the viral RNAs were purified and separated by 2-D polyacrylamide gel electrophoresis as previously described (37). ^{32}P-labeled cellular RNAs were prepared from secondary cultures of normal chicken embryo fibroblasts also as described previously (39). Figure 1 presents the 2-D gel analysis of the free small RNAs of polymerase-positive and polymerase-negative virions. Figure 1a shows the typical pattern of small RNAs from a nondefective avian sarcoma virus, SR-RSV-B. It is included to show that the glycoprotein defect of BH-RSV(−) does not affect the selection of small RNAs. As can be seen from Fig. 1b, the pattern for BH-RSV is identical to that of SR-RSV-B and previously published patterns (37, 39). In addition, quantitation of individual spots from gels of BH-RSV small RNAs showed no differences in the amounts of each small RNA relative to 70S RNA when compared to those obtained from SR-RSV (37). The small RNAs from the polymerase-negative virions of BH-RSVα form a distinctly different

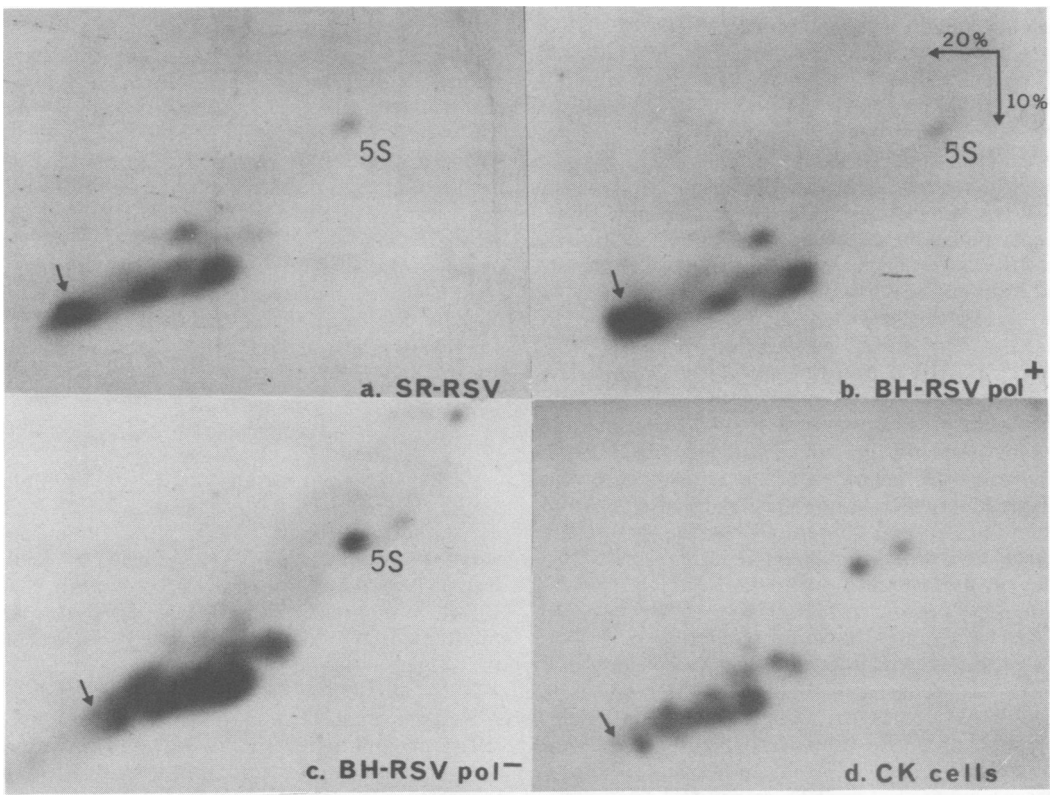

FIG. 1. *Small RNAs of polymerase-positive and polymerase-negative virions of RSV. Uniformly ^{32}P-labeled RNA was prepared from SR-RSV, BH-RSV, BH-RSVα, and uninfected chicken cells as described in the text. The 4 to 7S-size RNAs were separated by 2-D polyacrylamide gel electrophoresis. The first dimension (vertically in the figure) was in 10% acrylamide for 3 h at 400 V and 15°C. The second dimension was in 20% acrylamide for 18 h at 400 V and 15°C. The arrows indicate a 4S RNA that has been identified as the major in vitro primer for viral RNA-dependent DNA synthesis ($tRNA_{TRP}$). (a) SR-RSV; (b) BH-RSV; (c) BH-RSVα; (d) chick cell small RNAs.*

pattern (Fig. 1c). The autoradiogram contains numerous spots not seen in the pattern of polymerase-positive virus and is essentially identical to that for normal chick cell small RNAs (Fig. 1d). Thus, although BH-RSVα virions do incorporate some small RNA it appears that when the polymerase molecule is absent total cellular small RNA is packaged in a nonspecific manner. The spots indicated by the arrow in each figure were identified as $tRNA_{TRP}$ by RNase T1 fingerprint analysis (6, 13, 21, 37) (data not shown). Whereas $tRNA_{TRP}$ is a major species in polymerase-positive virions, it is only a minor species in BH-RSVα, in agreement with a previous estimate that $tRNA_{TRP}$ accounts for about 2% of cellular 4S RNA (21).

70S-associated small RNAs. The 70S genomic RNA is a complex of 35S RNA subunits and a small amount of 4 to 7S RNA. These 70S-associated small RNAs can be released by heating. When analyzed by 2-D gel they were found to be a subset of the small RNAs that can be found unassociated with 70S RNA (37). That is, all species found associated with the 70S RNA are also found in the free small RNA population, but several species of small RNA are only found free, never associated with the 70S RNA. Since the available evidence suggests that all virion small RNA is free in the particle at the time of budding and that the 70S-associated small RNAs become associated with genomic RNA, perhaps because of sequence homology, during a maturation process after budding (4, 5), it was of interest to determine what, if any, small RNAs were associated with BH-RSVα 70S RNA. ^{32}P-labeled 70S RNA was purified from BH-RSV and BH-RSVα, and the associated small RNA was analyzed by 2-D gels. In this procedure, only RNA of 7S or smaller enters the 10% acrylamide first dimension gel (37). The HMW RNA remains at the origin. Figure 2a shows an autoradiogram of a 2-D gel of BH-RSV 70S-associated small RNAs. It is identical to the previously published pattern for SR-RSV (37, 39). The arrow indicates the primer RNA, $tRNA_{TRP}$, which is present in about two copies per 70S RNA. This quantitation was done as described in Materials and Methods. All the other small RNAs are present in amounts less than one copy per 70S RNA. Figure 2b shows the 70S-associated small RNAs from BH-RSVα. Comparison of the two autoradiograms shows that all of the major spots in the 2-D gel of BH-RSV 70S-associated small RNAs are also found in the BH-RSVα 70S-associated small RNAs. However, whereas $tRNA_{TRP}$ is the major species of 70S-associated small RNA of BH-RSV, as judged by the intensity of the spot in the autoradiogram and by actual measurement of ^{32}P cpm in the gel, in BH-RSVα $tRNA_{TRP}$ is only a barely detectable spot on the autoradiogram (Fig. 2b, arrow). We were unable to prepare sufficient amounts of BH-RSVα 70S-associated small RNAs to obtain a reliable estimate of the number of copies of $tRNA_{TRP}$. Thus, BH-RSVα genomic RNA has associated with it the same species of small RNA as does 70S RNA from polymerase positive virions and is able to select the "correct" molecules. However, the amount of $tRNA_{TRP}$ relative to other species is greatly reduced.

Small RNAs of different species. The polymerases of two different species of avian retroviruses, the ALSV and the REV, are not closely related serologically, nor are the REV able to supply a functional polymerase to BH-RSVα (16, 24, 26–28, 38). Since it appears that the polymerase plays a major role in selecting small RNAs for packaging into virions, retroviruses having different polymerases might have different sets of small RNA. REV was grown on gs^-chf^- chicken embryo fibroblasts and thus, presumably, had the same population of host small RNAs from which to select the virion small RNAs. The autoradiograms of 2-D gel patterns for REV free and 70S-associated small RNAs are shown in Fig. 3. The patterns for ALSV and REV free small RNAs are distinct from each other and distinct from cellular small RNA. Likewise, the patterns of ALSV and REV 70S-associated small RNAs (Fig. 3b), are quite distinct from each other. This would be expected since association of small RNA with HMW RNA may be due to sequence complementarity, and the HMW RNAs of these two species show little, if any, homology (24).

DISCUSSION

Packaged within virions of RSV is a population of small RNAs including host cell tRNA, 5S rRNA, and 7S RNA (1–3, 7, 8, 11, 12, 34, 36). Most, if not all, cellular tRNA species are probably incorporated into virions to some extent. As shown by amino acid-tRNA acylation experiments, virion low-molecular-weight RNA can be charged with almost every amino acid (29, 33, 36, 40, 41, 43). However, only about eight tRNA's as well as 5S rRNA and 7S RNA are present in sufficient quantities to be detected as major spots in the autoradiograms of 2-D gel electrophoresis of ^{32}P-labeled RNA (37). These small RNAs are of host cell origin but represent a very specific and reproducible subset of total cellular small RNA (39). Until now little was known about the mechanism of this selection process. Homology with the virion genomic RNA probably does not play a role in the initial selection,

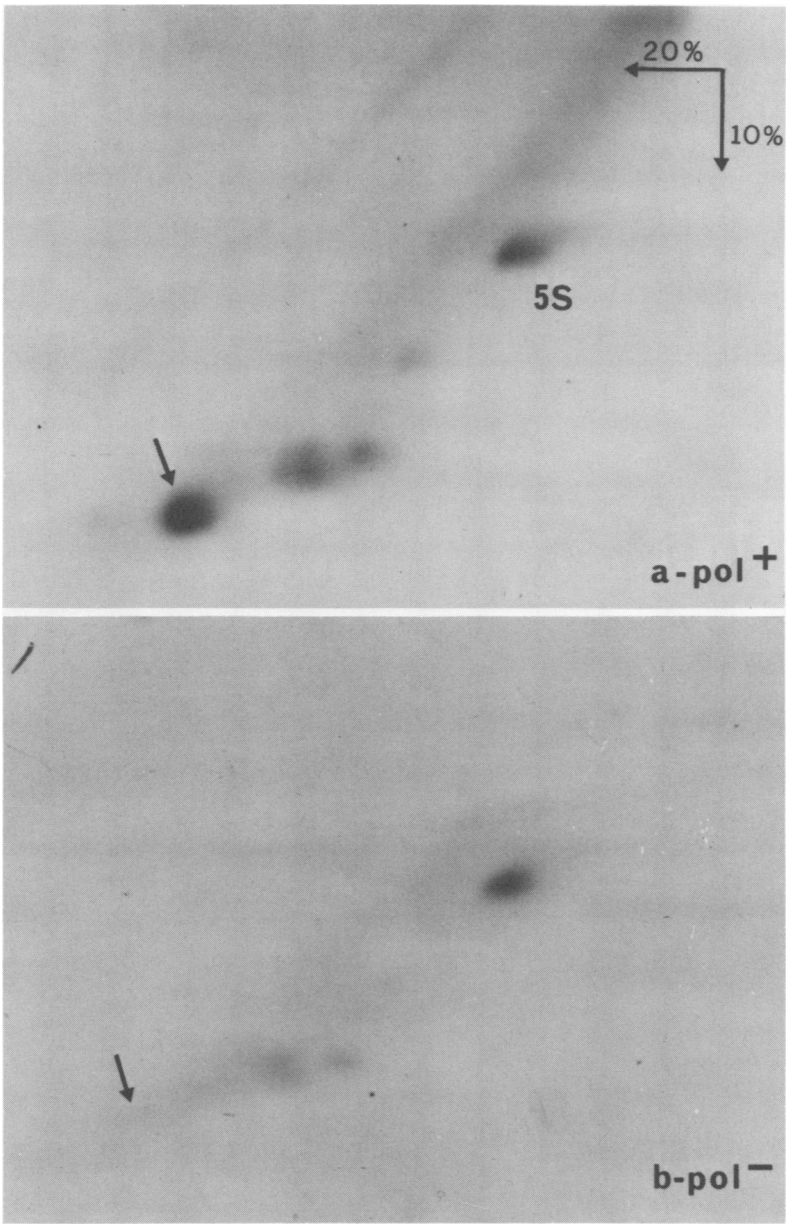

FIG. 2. *2-D separation of 70S-associated small RNAs of polymerase-positive BH-RSV and polymerase-negative BH-RSVα. Uniformly ^{32}P-labeled RNA was prepared from BH-RSV and BH-RSVα as described in the text. 70S RNA was isolated by sucrose gradient centrifugation. The 70S-associated small RNAs were released by heating (85°C, 3 min) and then analyzed by 2-D polyacrylamide gel electrophoresis as described in the text and Fig. 1. The arrows indicate $tRNA_{TRP}$, the major primer molecule for in vitro viral RNA-dependent DNA synthesis. (a) BH-RSV; (b) BH-RSVα (polymerase negative).*

since all the small RNA appears to be free in the virion right after budding (4, 5). Association of small RNA with the genomic RNA is thought to take place concurrent with the conversion to a 70S species, a process that occurs after budding for tissue culture-grown virus. Likewise, several small RNAs of RSV are found only free in the virion, never associated with the 70S RNA (37).

One of the virion small RNAs, a cellular $tRNA_{TRP}$, is the most abundant small RNA in ALSV virions and has been identified as the major primer for in vitro RNA-dependent DNA

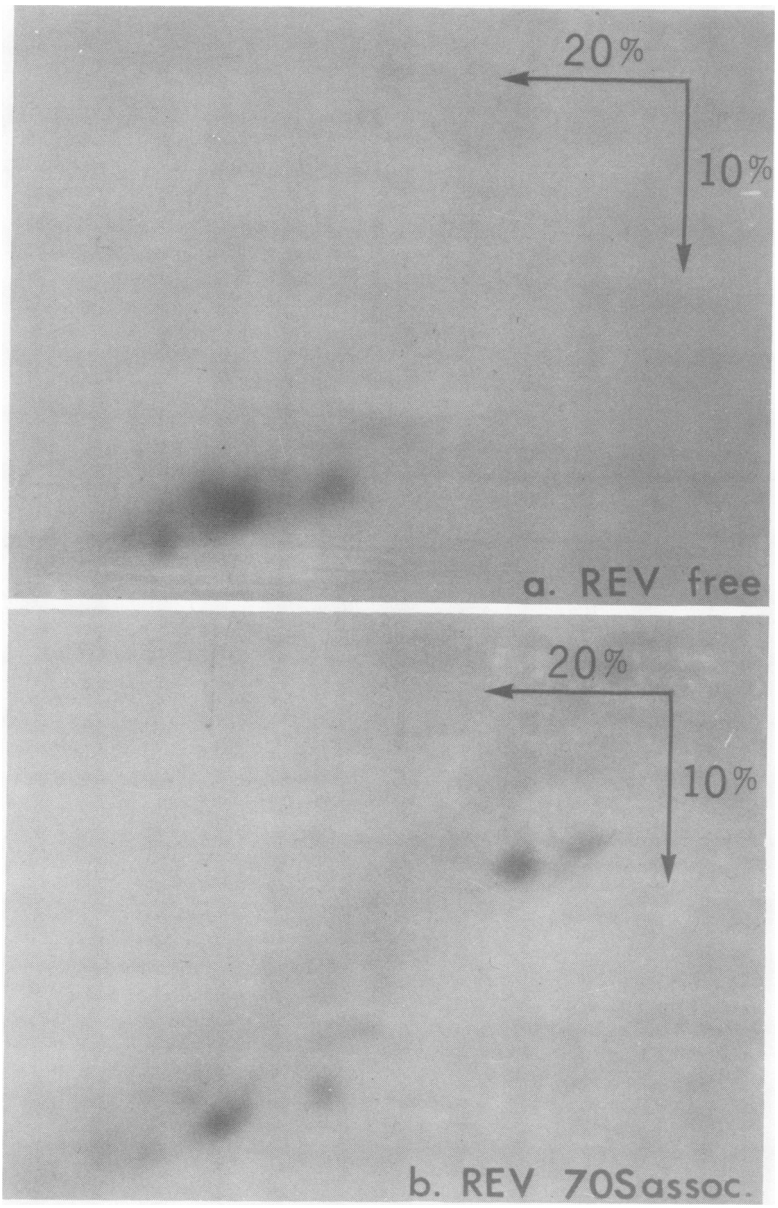

FIG. 3. *Small RNAs of REV. Uniformly ^{32}P-labeled RNA was prepared from virions of REV. The free small RNAs were separated from 70S RNA by sucrose gradient centrifugation. Free small RNAs and 70S-associated small RNAs were separated by 2-D polyacrylamide gel electrophoresis as described in the text and Fig. 1. (a) REV free small RNAs; (b) REV 70S-associated small RNAs.*

synthesis (6, 13, 15, 21, 39). If it serves this function in vivo, then its presence in the virion would be a biological necessity, requiring the virus to develop some mechanism for preferentially packaging a specific tRNA species that comprises no more than 2% of total cellular tRNA (21). The data obtained in this study strongly suggest that this mechanism is specific binding by the polymerase. Three lines of evidence support this conclusion. First, purified avian myeloblastosis virus polymerase, when mixed with total chicken cell tRNA, bound only $tRNA_{TRP}$, its primer RNA, and a small amount of $tRNA_{f\text{-}met}$, one of the other six major tRNA species found in avian myeloblastosis virus and RSV virions (30). Second, examination of the small RNAs of virions lacking polymerase protein revealed both a reduced amount of small

RNA and nonpreferential packaging of total host cell small RNA. Third, an unrelated species of avian retrovirus, REV, has a quite different small RNA composition.

The 2-D gel pattern of small RNAs of BH-RSV, the polymerase-positive form, were indistinguishable from those of nondefective SR-RSV-B and D and two different leukosis viruses, RAV-1 and RAV-2. However, the pattern of the small RNAs of BH-RSVα resembles not that of BH-RSVbut total cell tRNA. $tRNA_{TRP}$ is one of the predominant species found in polymerase-positive virions, whereas it is but a minor component in BH-RSVα, no more than is found in the cell. Thus, in the absence of polymerase molecules, RSV virions will still package some small RNA, but this RNA is an unselected mixture of total cellular tRNA. As judged by the intensity of spots in the autoradiograms, the amount of 5S RNA relative to 4S RNA is increased in BH-RSVα as compared to BH-RSV or SR-RSV, more closely resembling the relative amounts seen in cellular small RNA.

When we examined the 70S-associated small RNAs of polymerase-positive and polymerase-negative virions we observed that both populations contained the same species, but while the nonprimer-associated small RNAs of BH-RSVα seemed to be present in about the same proportion as in BH-RSV or SR-RSV, the amount of primer was greatly reduced. This may be due to the lack of enrichment of the free small RNAs for primer such that the very small amount of primer (about 2% of the BH-RSVα small RNA) is just not enough to saturate primer binding sites on the genomic RNA. The defect in BH-RSVα is unlikely to involve the primer binding site on the genomic RNA, since BH-RSVα 70S RNA readily serves as a template for reverse transcriptase following pseudotype formation (15, 16).

But what of the other small RNA species? No role requiring their specific incorporation into virions has been postulated. Nevertheless, they are specifically packaged only when the polymerase is present, suggesting that the enzyme is involved in their selection. Whereas the original polymerase-tRNA binding studies (31) indicated that the enzyme only bound $tRNA_{TRP}$ in vitro, recent work by Panet and Berliner (30) showed that under different assay conditions avian myeloblastosis virus polymerase will bind other tRNA's from chicken liver, yeast, or *Escherichia coli*. Sequence analysis beyond the RNase fingerprinting stage might reveal some structural features held in common as a possible explanation. An example is the replacement of the usual tRNA sequence $G_rT\psi CG$ with $G\psi\psi CG$. This sequence has been detected so far only in the two tRNA's that serve as primers for a retrovirus RNA-dependent DNA polymerase: $tRNA_{TRP}$ for ALSV and $tRNA_{PRO}$ in murine leukemia virus (MuLV)/murine sarcoma virus (22, 32).

That the packaging of small RNAs is a virus-controlled process is further substantiated by the observation that two different species of avian retroviruses, the ALSV and REV, when grown in chicken embryo fibroblasts each have quite different 2-D gel patterns of small RNAs. A third distinct population of virion small RNAs is obtained from MuLV. The 2-D gel patterns of MuLV free and 70S-associated small RNAs are distinct from those of ALSV or REV. The MuLV used in these studies was grown on 3T3 cells (32). However, the 2-D gel patterns of mammalian 4S RNAs (mouse, rat, and human) are virtually indistinguishable from those of avian species (39), suggesting that MuLV selects its small RNA from much the same population of cellular RNAs as do the avian species. This is consistent with this process being under virus rather than cellular control. Further, actinomycin D particles of MuLV, which lack HMW RNA but do contain polymerase, still contain the normal MuLV small RNA population (J. Levin, personal communication).

Although the primer RNA(s) for the REV is unknown, it seems unlikely to be $tRNA_{TRP}$ since this is a minor species in this virus. (In the two viruses where a primer has been identified, the primer is a major component of the small RNAs [32, 37]). This represents a possible partial explanation as to why this species, while able to supply a functional glycoprotein for the avian sarcoma virus (BH-RSV), is not able to rescue the polymerase-negative α form (38). REV polymerase might get packaged into BH-RSVα virions but would not pull in the correct primer, $tRNA_{TRP}$, making formation of a 70S RNA-enzyme-primer complex impossible.

Lack of proper primer molecules cannot be the whole explanation for the lack of complementation by different avian retroviruses for polymerase (38). It is, of course, possible that REV polymerase is simply not packaged into BH-RSVα virions, perhaps due to the significant size difference between the ALSV and REV enzymes (28).

Studies of rapid (3-min)-harvest virions have shown a number of changes taking place after budding: the viral core goes from electron lucent to electron dense; HMW RNA changes from 40 to 50S to 60 to 70S form; small RNAs associated with the HMW RNA and its ability to act as a template for reverse transcriptase increase (4, 5). This maturation process very well may be the formation of an HMW RNA-enzyme-primer initiation complex perhaps in some form as sug-

gested by Haseltine et al. (22). However, these results would appear to exclude the primer as a subunit linker, at least in the case of BH-RSVα, since these particles do contain a 65 to 70S RNA structure. Rather than an already hybridized primer functioning as a location marker for the enzyme, the enzyme may, in fact, play an active role in positioning the primer and the joining together of the subunits in a 5′-5′ linkage.

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