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Low-Molecular-Weight RNAs and Initiation of RNA-Directed DNA Synthesis in Avian Reticuloendotheliosis Virus

GORDON G. PETERS* AND CAROLINE GLOVER
Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, WC2A 3PX, United Kingdom

The small RNAs of avian reticuloendotheliosis virus (REV) were analyzed by two-dimensional polyacrylamide gel electrophoresis and compared with those of murine leukemia virus and avian sarcoma virus. Although there were some similarities among the three virus types, the patterns of small RNAs were distinct. By characterizing the small RNA which is most tightly associated with REV genome RNA and which can be labeled in limited DNA synthesis reactions, the primer for REV reverse transcription was identified as $tRNA^{Pro}$. This is consistent with previous reports that REV is more closely related to retroviruses of mammalian origin than to other avian viruses. In contrast, REV strong-stop complementary DNA is longer than any previously characterized strong-stop products of avian or mammalian retroviruses. The REV group may, therefore, have been derived from an as yet unidentified mammalian type C virus.

Reticuloendotheliosis virus (REV) is the prototype of a group of related, avian, type C retroviruses which cause a variety of lesions in infected birds (for a review, see reference 34). REV itself is capable of oncogenic transformation, inducing acute reticuloendothelial cell proliferation principally in the liver and spleen, whereas other symptoms associated with viruses of this group include anemia, spleen necrosis, and lymphoid cell infiltration of peripheral nerves (33, 34). In common with the other major group of avian retroviruses, the avian leukosis and sarcoma virus complex (ALSV), REVs appear to include both replication-defective acutely oncogenic viruses and their associated, less or nononcogenic helper viruses (17). However, in contrast to the ALSV group, REVs do not appear to be related to any endogenous type C retrovirus information in avian hosts (21). Moreover, REVs are quite distinct serologically, show little or no nucleic acid homology with members of the ALSV group, and neither genetically complement nor interfere with members of the ALSV group (12, 20, 22, 23, 32, 38, 48). Several studies on the morphology, major structural polypeptides, and DNA polymerase of REVs, presumably focusing on associated helper virus, have suggested that they are more closely related to type C viruses of mammalian origin than to the ALSV group (1, 2, 18, 25, 27, 51).

REV, like other retroviruses, replicates via a DNA intermediate which becomes integrated into the chromosomal DNA of the host cell (4, 11, 47). Thus, an obligatory step in replication is the reverse transcription of the viral RNA into DNA by the virion-associated DNA polymerase. The initiation of retrovirus DNA synthesis appears to be primed by one of the several cellular tRNA's found associated with the genome in the viral 70S RNA complex (6, 10, 31). This primer tRNA is bound to the genome RNA by a short region of base complementarity near the 5′ end of the genome (5, 8, 30, 42, 46). When viral 70S RNA is used to direct DNA synthesis either in disrupted virions or in reconstructed reactions catalyzed by purified reverse transcriptase, the initial piece of DNA made is a runoff product, representing a copy of the RNA between the primer binding site and the 5′ end of the genome (3, 14-16, 41, 43). This product has been referred to as "strong-stop complementary DNA" (strong-stop cDNA) (14, 15).

In the two well-characterized systems, Rous sarcoma virus (RSV) and Moloney murine leukemia virus (M-MuLV), the primers have been identified as $tRNA^{Trp}$ and $tRNA^{Pro}$, respectively, and the respective strong-stop cDNA's are 101 and 135 nucleotides long (6, 12a-14, 31). In view of the many similarities between avian REV and type C viruses of mammalian origin, we were interested in determining whether REV conforms to the avian or murine patterns or neither at the level of initiation of DNA synthesis. Here we present an analysis of the tRNA's associated with REV virions, an identification of the primer tRNA, and an estimation of the length of the strong-stop cDNA.

MATERIALS AND METHODS

Cells and viruses. A cell line chronically producing REV and free of detectable avian leukosis virus was

obtained by infecting a chemically transformed quail fibroblast line, QT35 (26), with REV-T (33, 34). The cells and original virus stock were generously provided by M. J. Hayman. Cells were routinely propagated in Dulbecco-modified Eagle medium supplemented with 10% tryptose phosphate, 4% fetal calf serum, and 1% dimethyl sulfoxide. M-MuLV was grown in a cloned line of NIH mouse 3T3 producer cells as previously described (9, 31). A line of transformed quail cells, designated 16Q, was used as a source of the Bryan high-titer strain RSV (BH-RSV) (28).

For the large-scale preparation of virus, the infected cells were grown in roller culture (Corning 490-cm^2 plastic bottles) in medium containing [5-^{3}H]uridine (1 μCi/ml) as a radioactive tracer. Every 24 h, virus was harvested from the culture fluid by high-speed centrifugation and purified by equilibrium sedimentation in sucrose density gradients (37).

Preparation of ^{32}P-labeled RNA. For ^{32}P labeling, cells were grown in phosphate-free Eagle medium supplemented with 2.5% dialyzed fetal calf serum, 1% dimethyl sulfoxide, and 0.2 mCi of $^{32}PO_4$ per ml. After labeling for 24 h, the medium was collected and replaced with unlabeled medium containing 0.1 mM sodium phosphate for a further 24 h. In some experiments, this labeling regime was repeated, or alternatively cells were grown in medium containing 0.1 mM phosphate and 0.1 mCi of $^{32}PO_4$ per ml for up to four 24-h periods. Virus was harvested from the pooled culture fluids by high-speed centrifugation, and the viral RNA was prepared by standard procedures (31, 37). Fractionation of viral RNA on sucrose gradients to prepare the 70S and free 4S RNA components, and subsequently 35S genome RNA and associated 4S RNAs, has been described elsewhere (31).

Analysis of low-molecular-weight RNAs. The low-molecular-weight fractions of ^{32}P-labeled RNA were analyzed by electrophoresis in two dimensions in polyacrylamide slab gels (2-D gels) (19, 31, 37). The first dimension was in 10% acrylamide (from top to bottom in all figures); the second dimension was in 20% acrylamide (from right to left in the figures). In some experiments, a third electrophoresis dimension was performed in 16% acrylamide slab gels containing 7 M urea (12a, 30). The ^{32}P-labeled RNAs were located by autoradiography, eluted from the gels, and characterized by RNase T_1 digestion and fingerprinting according to published procedures (31, 36, 37).

DNA synthesis reactions. (i) Labeling of primer RNA. Purified REV was suspended at a protein concentration of 2 mg/ml in a 20-μl reaction containing 50 mM Tris-hydrochloride (pH 7.6), 50 mM KCl, 10 mM magnesium acetate, 5 mM dithiothreitol, 0.02% Nonidet P-40, 15 mM phosphocreatine, 10 μg of creatine phosphokinase per ml, and 1 μM [α-^{32}P]dATP (Radiochemical Centre, Amersham, United Kingdom; 2,000 to 3,000 Ci/mmol). After incubation at 37°C for 60 min, the reaction was terminated by the addition of 2 ml of buffer containing 10 mM Tris-hydrochloride (pH 7.6), 100 mM NaCl, 10 mM EDTA, 0.2% sodium dodecyl sulfate, and 50 μg of carrier RNA per ml. The products were recovered by phenol extraction and ethanol precipitation and analyzed by 2-D gel electrophoresis.

(ii) Synthesis of cDNA. The initial products of 70S RNA-directed DNA synthesis were examined both in disrupted virions (endogenous reaction) and in reconstructed reactions containing purified 70S RNA and DNA polymerase from avian myeloblastosis virus (obtained through the Office of Program Resources and Logistics, National Cancer Institute, Bethesda, Md.). The components of the reactions were as described above, with the addition of dCTP, dGTP, and dTTP each at 0.1 mM. The reactions, containing either 10 μg of purified virus or 2 μg of 70S RNA plus 1 U of reverse transcriptase in a total volume of 5 μl, were incubated at 37°C for 60 min. To overcome possible chain termination of the nascent DNA due to the limiting concentration of [α-^{32}P]dATP, we added unlabeled dATP (0.1 mM) 15 min before stopping the reaction. The products were then recovered by phenol extraction and ethanol precipitation, and a portion of each was treated with pancreatic RNase ([illegible] μg/ml) for 30 min at 37°C to remove the RNA primer.

The ^{32}P-labeled cDNA products prepared in this way were analyzed by electrophoresis in one dimension in 0.3-mm-thick slab gels of 8% acrylamide containing 7 M urea. The samples were mixed with an equal volume of formamide and heated to 90°C for 2 min before loading on the gel. Electrophoresis was carried out in 50 mM Tris-borate buffer (pH 8.3) containing 1 mM EDTA for approximately 4 h at 1.5 kV. The gels were then wrapped in plastic film and exposed to Kodirex X-ray film (Kodak Limited, Herts, United Kingdom) at −70°C.

To obtain size estimates for the various cDNA products, we included a series of terminally labeled fragments of polyoma DNA (kindly provided by J. Arrand) as standards during electrophoresis. These DNA fragments had defined sequences corresponding to 275, 173, 112, and 46 nucleotides. In most experiments, ^{32}P-labeled 5S rRNA (120 nucleotides) and $tRNA^{Pro}$ (75 nucleotides) were included as additional size markers. A linear relationship was observed between the electrophoretic mobility of each marker and the logarithm of its chain length (see Fig. 4).

RESULTS

Free 4S RNAs of REV. (i) Analysis by 2-D gel electrophoresis. RNA prepared from ^{32}P-labeled REV was fractionated by sucrose gradient sedimentation to separate the 70S RNA complex from the free, low-molecular-weight RNAs. The small RNAs were then analyzed by electrophoresis in two dimensions in polyacrylamide slab gels (Fig. 1a). Only the 4S region of such a 2-D gel is depicted, but small amounts of molecules in the 5 to 8S size range were also observed, as reported for other retroviruses (31, 37). As shown schematically in Fig. 1b, about 10 distinct fractions were identified and numbered arbitrarily. The numbering system bears no relationship to those employed in the analogous characterization of the 4S RNA populations from M-MuLV and RSV (31, 37). The 2-D gel patterns for these latter two viruses are shown

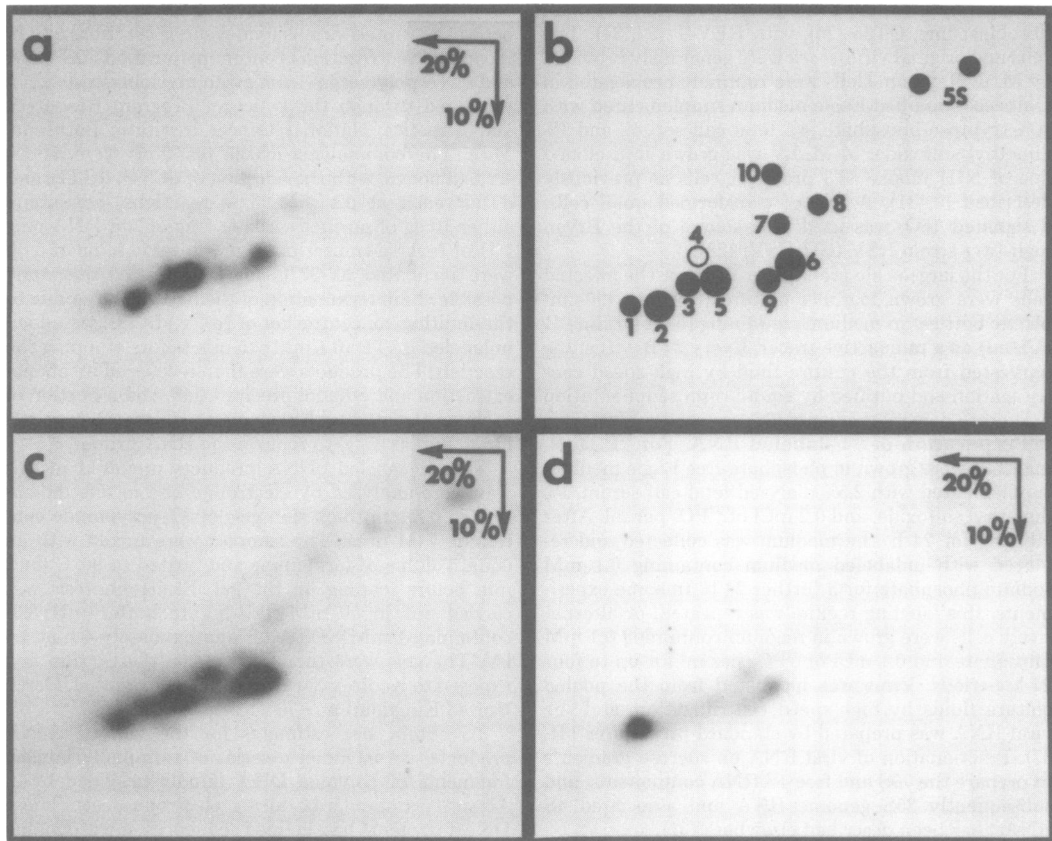

FIG. 1. *Comparison of free small RNAs of REV, M-MuLV, and RSV. ^{32}P-labeled viral RNA was prepared from REV-T, M-MuLV, and BH-RSV and separated into the 70S and free 4S RNA components by sucrose gradient centrifugation (31). The free 4S RNAs were then fractionated by 2-D gel electrophoresis and visualized by autoradiography as previously described (19, 31, 37). (a) Free 4S RNAs from REV-T; (b) schematic drawing of REV small RNA pattern, showing numbering system used; (c) free 4S RNAs from M-MuLV; (d) free 4S RNAs from BH-RSV.*

in Fig. 1c and d, respectively. Although there were some similarities among the free 4S RNAs of REV, M-MuLV, and RSV as analyzed by 2-D gel electrophoresis, the three patterns were clearly distinct from one another. A more reliable comparison of the small RNAs found in the different viruses was obtained by RNase T_1 digestion and fingerprinting of the various RNA species identified as "spots" on the 2-D gel autoradiograph.

(ii) Fingerprints of individual RNAs. The RNAs corresponding to the numbered spots in Fig. 1b were eluted and characterized by RNase T_1 digestion and oligonucleotide fingerprinting. In several cases, the complexities of the fingerprints obtained were indicative of mixtures of at least two 4S RNAs. These could generally be resolved by a further dimension of electrophoresis in gels containing urea (30). The fingerprints of the major 4S RNAs of REV, numbered as in Fig. 1b and further resolved on urea gels, are shown in Fig. 2.

Several of the fingerprints corresponded to previously characterized tRNA species which are common to all three of the viruses under discussion. For example, the RNA in spot 1 had the fingerprint of $tRNA^{Trp}$, the primer for RSV reverse transcription, whereas spot 5 RNA was $tRNA^{Pro}_{1+2}$, the primer for M-MuLV (12a, 13). In addition, spot 2a RNA (as yet uncharacterized), spot 2b RNA ($tRNA^{Gly}$ [J. Dahlberg, personal communication]), and spot 6b RNA ($tRNA^{Lys}_{1+2}$ [35; Peters and Glover, manuscript in preparation]) are major species common to REV, M-MuLV, and RSV (31, 37).

Identification of primer RNA for REV DNA synthesis. (i) 4S RNAs associated with REV genome. Thermal denaturation of retro-

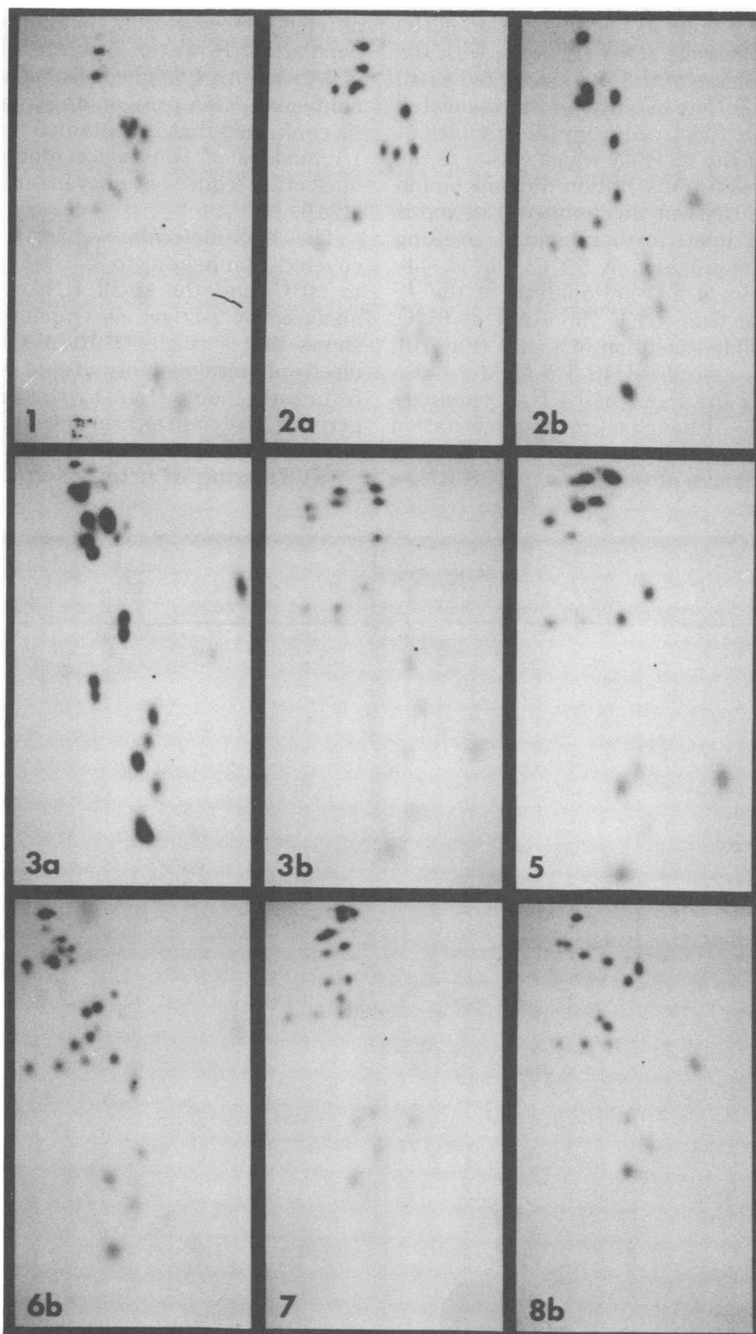

FIG. 2. *RNase T_1 fingerprints of individual REV 4S RNAs. RNA species corresponding to the numbered spots in Fig. 1b were resolved further by electrophoresis in a 16% acrylamide gel containing 7 M urea (30). The repurified RNAs were then eluted from the gel and analyzed by RNase T_1 digestion and oligonucleotide fingerprinting (31, 36, 37). The first dimension, from 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 paper in 7% formic acid. The size of the final fingerprint was approximately 15 by 25 cm.*

virus 70S RNA results in the dissociation of the two 35 to 40S genome RNA subunits, with the concomitant release of the 70S-associated small RNAs (6, 31, 37). The majority of the associated small RNAs are displaced from the complex at 60 to 65°C, but the 4S RNA which serves as the primer for reverse transcription remains bound to the genome RNA at this temperature and is only displaced upon further heating (melting temperature, approximately 75°C) (6, 30, 44). Figure 3a shows a 2-D gel analysis of the 4S RNAs released from REV 70S RNA at 65°C. With the possible exception of spot 4 (Fig. 1b), all of the 70S-associated 4S RNAs were also present among the free small RNAs (compare Fig. 3a and 1a). Although a precise quantitation was not performed, it was apparent that the relative abundances of the associated 4S RNAs differed somewhat from that of the free 4S RNA population. However, in both fractions, spot 5 RNA was clearly and consistently the predominant small RNA present, and fingerprint analysis confirmed that it contained only $tRNA^{Pro}_{1+2}$ (a 1:1 mixture of two isoacceptor species whose nucleotide sequences differ in only two positions [12a]).

The high-molecular-weight REV RNA recovered after heating to 65°C was further heated to 90°C, and the small RNAs released were analyzed by 2-D gel electrophoresis. Figure 3b shows that a single 4S RNA species with the electrophoretic mobility of spot 5 was displaced from the genome RNA by this treatment. Fingerprint analysis confirmed that it was, indeed, equivalent to spot 5 RNA, i.e., $tRNA^{Pro}_{1+2}$.

(ii) Tagging of primer with [α-^{32}P]dATP.

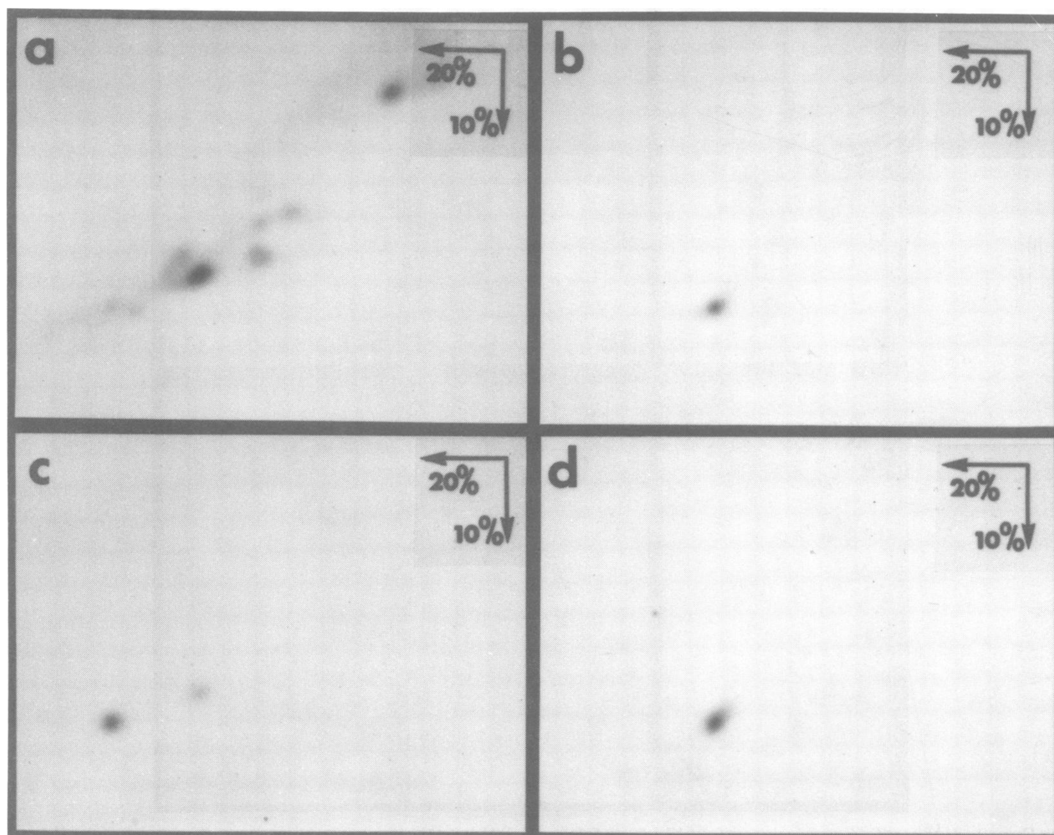

FIG. 3. *Identification of RNA primer for REV DNA synthesis. Uniformly ^{32}P-labeled REV 70S RNA was heat denatured in two steps. (a) Small RNAs released from the complex at 65°C were analyzed by 2-D gel electrophoresis. (b) High-molecular-weight RNA recovered after heating to 65°C was heated further to 95°C, and the small RNA released was analyzed by 2-D gel electrophoresis. (c and d) Unlabeled REV virions were used to direct DNA synthesis in a reaction containing [α-^{32}P]dATP as the only deoxynucleoside triphosphate. The single small RNA species labeled in such a reaction was purified by gel electrophoresis. (c) Co-electrophoresis of [^{32}P]dAMP-tagged primer (2,000 cpm) with uniformly ^{32}P-labeled $tRNA^{Trp}$ (4,000 cpm). (d) Co-electrophoresis of [^{32}P]dAMP-tagged primer (2,000 cpm) with uniformly ^{32}P-labeled $tRNA^{Pro}$ (4,000 cpm).*

In limited DNA synthesis reactions directed by retrovirus 70S RNA, molecules capable of acting as primers for reverse transcription acquire covalent extensions of DNA at their 3′ ends (7, 15, 45). If only a single α-^{32}P-labeled deoxynucleoside triphosphate precursor is supplied, corresponding to the first residue added to the primer, then molecules which function as primers can be specifically "tagged" with ^{32}P (6, 31). Purified REV was suspended in a DNA synthesis reaction containing [α-^{32}P]dATP, and the tagged primer RNA was isolated as described above. A single 4S RNA acquired ^{32}P label under these conditions. The terminally labeled primer (2,000 cpm of ^{32}P) was then co-electrophoresed with purified, uniformly labeled tRNA^{Trp} (4,000 cpm) or tRNA^{Pro} (4,000 cpm). As shown in Fig. 3c and d, the electrophoretic mobility of the tagged primer was quite different from that of tRNA^{Trp} (the primer is the fainter spot in Fig. 3c) but was indistinguishable from that of tRNA^{Pro}. Furthermore, the mixture of uniformly labeled and terminally labeled RNAs was eluted from the gel shown in Fig. 3d and analyzed by RNase T_1 fingerprinting (data not shown). The fingerprint contained two additional oligonucleotides corresponding to the 3′-terminal oligonucleotide of tRNA^{Pro} extended by either one or two dAMP residues (31).

REV strong-stop cDNA. When 70S RNA is used to direct DNA synthesis, the major product synthesized initially is a runoff product representing the distance between the tRNA binding site and the 5′ end of the genome. The length of this product, called strong-stop cDNA, appears to be characteristic of different classes of retrovirus (14). REV cDNA was synthesized in both endogenous reactions (disrupted virions) and reconstructed reactions containing purified 70S RNA and avian myeloblastosis virus DNA polymerase. The length of the major product, strong-stop cDNA, was determined by its electrophoretic mobility relative to a series of standards of defined chain length. Figure 4 shows the results obtained in a reconstructed reaction in which [α-^{32}P]dATP was the labeled precursor. Similar products were observed with alternative labeled precursors and in endogenous reactions. Confirmation that the putative strong-stop cDNA was covalently linked to a tRNA primer was obtained by RNase treatment of the reaction products before electrophoresis. In track a of Fig. 4, the major labeled product had a chain length of 250 nucleotides, based on the observed linear relationship between the electrophoretic mobility and the logarithm of the chain length of the size markers. Pretreatment with pancreatic RNase (Fig. 4, track b) reduced the major product to 178 nucleotides, consistent with the anticipated removal of 74 nucleotides of tRNA (the 3′-terminal adenosine on the tRNA would remain attached to the product after pancreatic RNase digestion). In several such experiments, the length of REV strong-stop cDNA was consistently computed to be 178 ± 2 nucleotides.

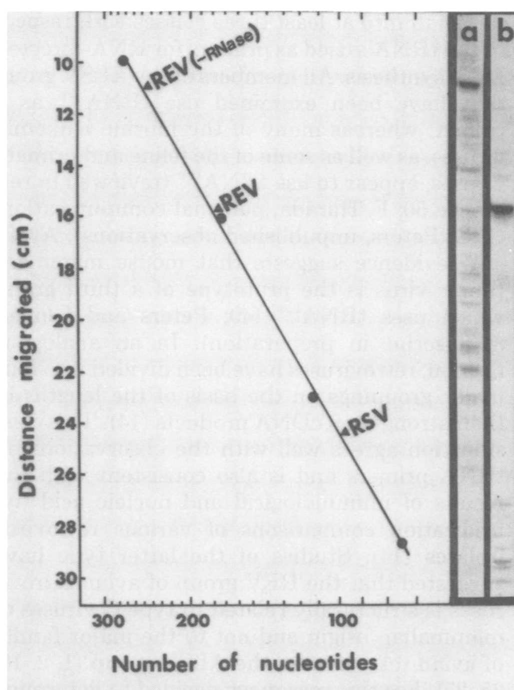

FIG. 4. *REV strong-stop cDNA. REV cDNA was synthesized in a reconstructed in vitro reaction containing REV 70S RNA, [α-^{32}P]dATP, unlabeled dCTP, dGTP, and dTTP, and purified reverse transcriptase as described in the text. The products were divided into two portions, one of which was treated with pancreatic RNase (200 μg/ml) to remove any RNA primer attached to the DNA product. Both samples were then denatured and analyzed by electrophoresis in an 8% acrylamide slab gel containing 7 M urea, along with a variety of size markers. (a) REV strong-stop cDNA; (b) REV strong-stop cDNA pretreated with RNase. The main panel of the figure shows the inverse logarithmic relationship between the electrophoretic mobility and the nucleotide chain length of the size standards. BH-RSV strong-stop cDNA analyzed on the same gel had the expected size of 101 nucleotides.*

DISCUSSION

Analyses of the events involved in the initiation of reverse transcription can yield important information regarding the structure of the 5′ end of retrovirus genome RNAs. To date, retrovi-

ruses fall into at least three classes with respect to the tRNA's used as primers for RNA-directed DNA synthesis. All members of the ALSV group that have been examined use $tRNA^{Trp}$ as a primer, whereas many of the murine leukemia viruses, as well as some of the feline and primate viruses, appear to use $tRNA^{Pro}$ (reviewed in reference 50; F. Harada, personal communication; G. G. Peters, unpublished observations). Available evidence suggests that mouse mammary tumor virus is the prototype of a third group which uses $tRNA^{Lys}$ (49; Peters and Glover, manuscript in preparation). In an analogous fashion, retroviruses have been divided into four major groupings on the basis of the lengths of their strong-stop cDNA products (14). This classification agrees well with the observations on tRNA primers and is also consistent with the results of immunological and nucleic acid hybridization comparisons of various retrovirus isolates (14). Studies of the latter type have suggested that the REV group of avian retroviruses is structurally related to type C viruses of mammalian origin and not to the major family of avian retroviruses, the ALSV group (1, 2, 18, 25, 27). For this reason we decided to determine whether REV conforms to any of the known patterns at the level of initiation of reverse transcription.

As with other retroviruses, REV contained a specific subset of the host cell tRNA population in the mature virion particles. It remains unclear how or why the virus selects these tRNA's, but REV consistently packaged the same 10 to 15 tRNA's in approximately the same relative proportions (39). Cloning of the virus by two successive endpoint dilutions did not alter the pattern of virion small RNAs (data not shown), confirming that the studies described here refer to nondefective, helper virus and not to a defective, oncogenic agent which was presumably present in the initial virus stock (17). Because of difficulties in resolving some of the 4S RNA species, a precise quantitation of the number of copies of each tRNA per virion was not obtained, but it was clear that several of them were present in less than one copy per virion. Unless the virus population was heterogeneous with regard to some aspects of its life cycle, which was unlikely in the cloned virus stock, it is difficult to envisage a function for these minor tRNA's.

Several of the REV tRNA's whose structures are highly conserved among different eucaryotic species, judging from fingerprint analysis, were found to be common to REV, M-MuLV, and RSV. It has been suggested that the selection of tRNA's for inclusion in virus particles is determined to a large extent by the viral RNA-directed DNA polymerase (29, 39). However, although REV DNA polymerase has a structure and divalent ion preference similar to those of the MuLV enzyme (24, 25, 32; data not shown), the patterns of REV and M-MuLV small RNAs were quite distinct. In fact, REV could not be said to conform to either RSV or M-MuLV patterns in the selection of free or associated 4S RNAs. Nevertheless, REV did resemble M-MuLV in that $tRNA^{Pro}$ was the most abundant species in the free and associated 4S RNA fractions of both viruses (REV spot 5 and M-MuLV spot 6 RNAs) (31). In addition, $tRNA^{Pro}$ was the most tightly bound 70S-associated small RNA, and a molecule with the same electrophoretic mobility as that of $tRNA^{Pro}$ was the only small RNA labeled with [α-^{32}P]dATP in a limited DNA synthesis reaction directed by REV 70S RNA. Although not rigorous proof, these results taken together clearly indicated that the major primer for REV DNA synthesis, at least in vitro, was $tRNA^{Pro}$.

Thus, REV, although isolated from avian hosts, could be classified as belonging to the large group of mammalian viruses which use $tRNA^{Pro}$ as the primer for reverse transcription. However, in contrast to these findings, the length of REV strong-stop cDNA (178 nucleotides) did not conform to any previously described classes. Moreover, preliminary sequence analysis of this product has not revealed any homologies with either ALSV or M-MuLV strong-stop cDNA's (data not shown). Therefore, the possibility remains open that REV was, indeed, originally derived from a mammalian virus, but one which has not yet been identified or fully characterized. If, on the other hand, REV had been acquired very early by its present-day avian hosts and had been altered from its original mammalian form by recombination and mutation, one would not expect the various isolates of the REV group to be so similar. They might also have been expected to acquire genetic information of avian origin in the course of evolution, but this does not seem to be the case (21). In fact, no REV-specific sequences have been detected in the DNAs from a wide variety of avian species or in mouse or human DNA (D. Frisby, personal communication). Therefore, REV is one of the few examples of infectious retroviruses which show no detectable genetic homology with cellular DNA of their natural hosts. Whether this statement will also apply to the defective, oncogenic component of REV is an intriguing question. The defective acute leukemia viruses of the ALSV group appear to be recombinants between nondefective leukosis viruses and some host cell sequences containing a

potential oncogene (40; D. Stehelin, S. Saule, M. Roussel, C. Lagron, and C. Rommens, Cold Spring Harbor Symp. Quant. Biol., in press). A similar situation might occur in REV but may have gone undetected because of a vast excess of nondefective helper in the virus stocks. If this is the case, it will be interesting to determine whether the oncogenic information, like the helper, is of mammalian rather than avian origin.

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