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RNA-Directed DNA Synthesis in Moloney Murine Leukemia Virus: Interaction Between the Primer tRNA and the Genome RNA

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Initiation of RNA-directed DNA synthesis in virions of Moloney murine leukemia virus requires a cellular $tRNA^{Pro}$ as primer. The site(s) on the Moloney murine leukemia virus genome RNA at which functional primer molecules are bound and at which purified $tRNA^{Pro}$ hybridizes has been located near (within 20%) the 5′ end of the genome. A relatively stable duplex (temperature at which 50% dissociation has occurred, 76°C) is formed between the amino acid acceptor stem of the $tRNA^{Pro}$ and a complementary sequence in the Moloney murine leukemia virus 35S RNA. The interaction involves 19 base pairs, extending from the penultimate nucleotide at the 3′ end of the $tRNA^{Pro}$ but apparently not including the 3′-terminal adenosine residue. In most respects, the interaction between primer and template in Moloney murine leukemia virus parallels the situation in the avian leukosis-sarcoma viruses.

Synthesis of DNA in vitro by the RNA-directed DNA polymerase of RNA tumor viruses is initiated by elongation of a primer (18, 34, 35). When viral 70S RNA is used to direct DNA synthesis, either in detergent-disrupted virions or in reconstructed reactions with purified reverse transcriptase, one of the small RNAs associated with the genome in the 70S complex serves as the major primer (3, 17). In the two systems most rigorously studied, Rous sarcoma virus (RSV) and Moloney murine leukemia virus (M-MuLV), the primers have been identified as normal cellular tRNA's (10, 16, 17, 27, 29). These are $tRNA^{Trp}$ in RSV and $tRNA^{Pro}$ in M-MuLV (19, 36; F. Harada, G. Peters, and J. E. Dahlberg, J. Biol. Chem., in press).

The interaction between primer tRNA and template, the 35S genome RNA of the virus, is crucial in directing the initiation of DNA synthesis. We have examined the interaction between $tRNA^{Pro}$ and M-MuLV genome RNA to make a comparison with the analogous situation in RSV. The primer binding site was localized relative to the 3′ end of the genome RNA by determining the minimum size of polyadenylated viral RNA which can prime DNA synthesis (native complex) or which can reanneal to purified $tRNA^{Pro}$. Similar results were obtained with the two approaches. In addition, the native and reconstructed complexes had the same T_m for dissociation, i.e., temperature at which 50% dissociation had occurred. Interaction of primer and template resulted in the formation of a duplex which was resistant to hydrolysis by RNase under appropriate conditions. By isolating the duplex formed between radioactively labeled $tRNA^{Pro}$ and unlabeled M-MuLV 35S RNA, the nucleotide sequence responsible for the interaction was analyzed.

Our results for the interaction between $tRNA^{Pro}$ and the genome of M-MuLV are very similar to those previously reported for the interaction of $tRNA^{Trp}$ and the RSV genome (6, 13, 30, 33).

MATERIALS AND METHODS

Virus growth and isolation. M-MuLV was propagated in a cloned line of 3T3 producer cells, designated clone 1, according to published procedures (14, 15). Cells were routinely grown in Eagle medium supplemented with 5% fetal calf serum, and virus was harvested from the culture fluid at 24-h intervals. For preparing [^{3}H]RNA, [5-^{3}H]uridine (New England Nuclear Corp.; 26 to 28 Ci/mmol) was added to the medium at a final concentration of 0.04 mCi/ml. For ^{32}P labeling, cells were grown in phosphate-free medium supplemented with dialyzed serum and containing 0.5 to 1.0 mCi of $^{32}PO_4^{3-}$ per ml. Only one viral harvest was taken from ^{32}P-labeled cells 20 h after addition of label so that the cells could be used to isolate cytoplasmic RNA as described below. Virus was isolated from the culture medium by high-speed centrifugation and, when intended for use in DNA

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synthesis reactions, further purified by centrifugation to equilibrium in sucrose gradients (28).

Isolation of cellular and viral RNA. RNA was isolated from pelleted virus and fractionated into 70S and 4 to 7S components as previously described (27, 28). The 70S RNA was dissociated by heating to 95°C for 2 min, and the high-molecular-weight genome RNA was separated from the 70S-associated small RNAs by sedimentation through sucrose gradients (27). Genome RNA prepared in this manner from 24-h harvests of virus was heterogeneous in size, ranging from about 10S up to full-length 35S subunits.

To prepare cell RNAs, the medium was removed and the ^{32}P-labeled cells were washed with buffer containing 0.02 M Tris-hydrochloride (pH 7.8), 0.1 M NaCl, and 1 mM EDTA; cells were then treated on the culture plates with the same buffer containing 1% Triton X-100 (1 ml/10-cm dish) for 15 min at room temperature. The cell material was collected and centrifuged at 750 × *g* for 10 min to remove nuclei and debris. Nucleic acids were extracted from the cytoplasmic supernatant by standard procedures (30) and fractionated by sedimentation through 18-ml gradients of 5 to 20% sucrose in 0.02 M Tris-hydrochloride (pH 7.8)–0.1 M NaCl–1 mM EDTA–0.5% Sarkosyl. The gradients were centrifuged at 100,000 × *g* for 20 h at 4°C in a Beckman SW27 rotor, and the major 28S, 18S, and 4S cytoplasmic RNA fractions were recovered by ethanol precipitation.

For the preparation of [^{32}P]tRNA^{Pro}, the cellular 4S RNA was further fractionated by electrophoresis in polyacrylamide gels. An initial purification was achieved by using the two-dimensional gel system described by Ikemura and Dahlberg (23). After autoradiography, the region of the gel containing tRNA^{Pro} was identified by comparison with the pattern of small RNAs from M-MuLV virions (27). This segment was cut out and placed at the origin of a 16% acrylamide slab gel containing 7 M urea. Electrophoresis was carried out for 16 h at 350 V and 15°C, using the Tris-EDTA-borate buffer system described by Peacock and Dingman (26). The labeled tRNA was eluted from the gel and characterized as previously described (27, 28).

DNA synthesis reactions. Sucrose gradient-purified M-MuLV (2 to 3 mg) was concentrated by high-speed centrifugation and suspended in a 1 ml of reaction mixture containing 0.1 M Tris-hydrochloride (pH 8.4), 0.01 M magnesium acetate, 0.02 M 2-mercaptoethanol, 0.01% Nonidet P-40, 100 μg of actinomycin D per ml, and 2.5 μM [α-^{32}P]dATP (Amersham Corp.; 136 Ci/mmol). After incubation at 37°C for 1 h, viral RNA was extracted by standard procedures (28). Under these conditions, a single small RNA species, namely, tRNA^{Pro}, was labeled at its 3′ end with [^{32}P]dAMP (27). Viral 70S RNA recovered after the DNA synthesis reaction was heated to 65°C for 2 min, conditions which caused dissociation of the 35S RNA subunits but did not displace the primer from the genome RNA (27). The complex of primer and genome RNA prepared in this way was regarded as the "native" form to distinguish it from the "reconstructed" complex described below.

Annealing of primer to genome RNA. Mixtures of purified M-MuLV 35S RNA (either unlabeled or ^{3}H labeled) and [^{32}P]tRNA^{Pro} were precipitated in ethanol and dissolved in 0.6 M NaCl–0.02 M Tris-hydrochloride (pH 7.5)–0.02 M EDTA to obtain a final concentration of between 0.5 and 1.0 mg of 35S RNA per ml and 10 to 20 μg of tRNA^{Pro} per ml. If a single binding site for tRNA^{Pro} existed on the 35S RNA, then the tRNA was present in a two- to threefold molar excess in such mixtures. The solution was heated to 95°C for 2 min to disrupt inherent secondary structure in the RNA, cooled to 70°C, and maintained at this temperature for a further 20 min. The annealed primer-template complex (reconstructed complex) was separated from free tRNA^{Pro} by sucrose gradient sedimentation (27).

Poly(U)-Sepharose chromatography. Samples of ^{3}H-labeled genome RNA carrying primer which was either tagged with [^{32}P]dAMP (native complex) or uniformly labeled with ^{32}P (reconstructed complex) were separated into polyadenylic acid [poly(A)]-deficient and poly(A)-containing fractions by chromatography on polyuridylic acid [poly(U)]-Sepharose 4B (Pharmacia Fine Chemicals, Inc.) essentially as described by Eiden and Nichols (12). The RNA was first heated to 65°C for 2 min to disrupt aggregates of the genomic RNA subunits and diluted into 1 ml of 0.1 M NaCl–0.01 M EDTA–0.01 M Tris-hydrochloride (pH 7.5)–0.2% sodium dodecyl sulfate for application to the column. After thorough washing of the column with the same buffer, the polyadenylated RNA, representing about 30% of the total RNA applied, was eluted with distilled water. The poly(A)-deficient and poly(A)-containing fractions of RNA were recovered by ethanol precipitation.

Gel electrophoresis of primer-template complexes. Samples of the RNA recovered after poly(U)-Sepharose chromatography were analyzed by electrophoresis in slab gels of 2.25% acrylamide stabilized with 0.5% agraose by the procedure of Peacock and Dingman (26). To obtain size estimates for the various genome RNA preparations, the following [^{32}P]RNAs were included as standards during electrophoresis: M-MuLV virion 35S RNA, the 28S, 18S, and 5S rRNA's from 3T3 mouse cells, and the 30S rRNA precursor and mature 23S and 16S rRNA's from *Escherichia coli*. The *E. coli* RNAs were generously provided by E. Lund. The chain lengths of these marker RNAs were assumed to be 10,000, 6,000, 5,500, 3,300, 2,500, 1,550, and 120 nucleotides, respectively, in order of decreasing S value. In these gels, the electrophoretic mobility of the RNA was inversely proportional to the logarithm of its chain length (26).

After electrophoresis, the gels were fixed and stained in 50% formamide containing 0.01% Stains All (8). The ^{32}P-labeled primer was located by autoradiography. Partial drying of the gel was found to enhance the resolution of the autoradiograph, but total dehydration was avoided since it seriously reduced the efficiency of ^{3}H detection. The ^{3}H-labeled genome RNA was detected by scintillation counting of segments of the gel strips.

RNase digestion of primer-template complex. To determine the extent of the interaction between tRNA^{Pro} and the M-MuLV genome, the reconstructed complex containing [^{32}P]tRNA^{Pro} was digested with

ribonucleases under conditions in which duplex regions of RNA were resistant to hydrolysis. A variety of enzyme combinations was used, but in all instances the salt concentration was maintained at 0.3 M and the temperature was maintained at 25°C. The digestions were carried out in a final volume of 20 to 30 μl and in the presence of 50 μg of carrier RNA, using the following: (i) 5 μg of pancreatic RNase (EC 3.1.4.22; Worthington Biochemicals Corp.) in 0.01 M Tris-hydrochloride (pH 7.5)–0.01 M EDTA; (ii) 5 μg of RNase T_1 (EC 3.1.4.8; Calbiochem) in 0.01 M Tris-hydrochloride (pH 7.5)–0.01 M EDTA; or (iii) 5 U of RNase T_2 (EC 3.1.4.23; Calbiochem) in 0.1 M sodium acetate (pH 4.0)–0.01 M EDTA. T_1 and pancreatic RNase digestions, whether separate or combined, were carried out for 1 h, whereas hydrolysis by RNase T_2 was allowed to proceed for 20 h and always followed a prior treatment with either T_1 or pancreatic RNase. The resultant double-stranded fragments of RNA were isolated by electrophoresis in 20% acrylamide gels (23) and analyzed by conventional RNA fingerprinting and sequencing techniques (1, 2, 24).

RESULTS

Location of functional primer on the M-MuLV genome RNA. Molecules which function as primers for DNA synthesis in M-MuLV can be labeled at their 3′ termini by supplying detergent-disrupted virions with [α-^{32}P]dATP (27). The binding site of the functional primer(s) was located relative to the 3′ end of the genome by determining the minimum size of polyadenylated viral RNA which carried [^{32}P]dAMP-tagged primer molecules (30, 33). Results of such an experiment are shown in Fig. 1.

Figure 1a represents an autoradiograph obtained after gel electrophoresis of poly(A)-deficient and poly(A)-containing M-MuLV RNA on which functional primers had been labeled with [^{32}P]dAMP. With poly(A)-deficient RNA, the ^{32}P-tagged primer was distributed on all size classes of the genome RNA from about 10S up to full-length 35S molecules. In contrast, only a very restricted size class of polyadenylated RNA molecules carried the tagged primer. On the gel system used, this class of RNA was indistinguishable from full-length 35S RNA. These results are consistent with the location of functional primer very near the 5′ end of the genome RNA. Thermal denaturation of the complex of tagged primer and genome RNA revealed that a single 4S RNA species had been labeled in the DNA synthesis reaction (Fig. 1a). Further analysis (data not shown) confirmed that this primer was indistinguishable from $tRNA^{Pro}$ (27; Harada et al., J. Biol. Chem., in press).

Since the results shown in Fig. 1a could also be obtained if the polyadenylated fraction of the genome RNA contained only full-length 35S molecules, it was necessary to establish that

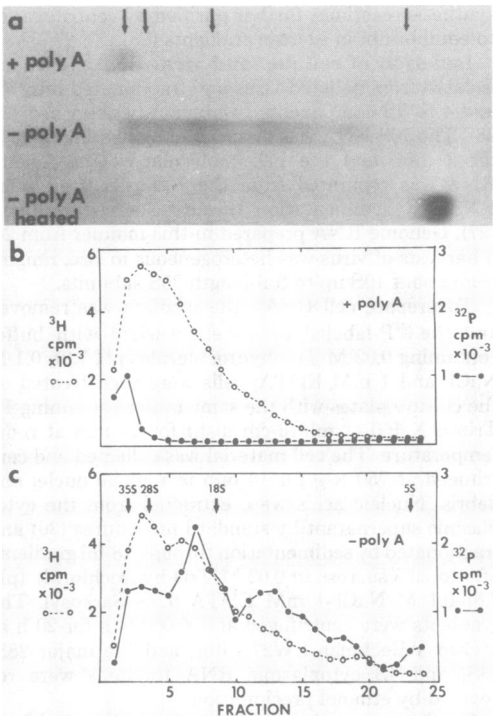

FIG. 1. *Location of functional primers on the M-MuLV genome. Detergent-disrupted M-MuLV, uniformly labeled with [^{3}H]uridine, was used to direct DNA synthesis with [α-^{32}P]dATP as the only deoxynucleotide precursor. The viral RNA was isolated under conditions which did not displace the primer from the genome and was separated into poly(A)-containing and poly(A)-deficient fractions. The two fractions were analyzed by electrophoresis in 2.25% acrylamide–0.5% agarose gels at 250 V for 3 h at 4°C (26). Electrophoresis was from left to right, and the arrows indicate the positions of the 35S, 28S, 18S, and 5S RNA included as markers in the gel. (a) Autoradiograph of the gel analysis of ^{3}H-labeled genome RNA carrying [^{32}P]dAMP-tagged primer. (b) Segments (0.5-cm) from the gel track of polyadenylated RNA were excised, and the ^{3}H and ^{32}P radioactivity in each was determined by scintillation counting. (c) Distribution of ^{3}H and ^{32}P radioactivity in poly(A)-deficient RNA.*

both fractions of genome RNA were heterogeneous in size. This was accomplished by prelabeling the viral RNA with [^{3}H]uridine. Figures 1b and c show the distribution of ^{3}H and ^{32}P in the corresponding gel tracks from (a). It is evident that the size distribution of the genome RNA was essentially the same for the poly(A)-deficient and poly(A)-containing fractions. Analyses of this type also permitted a more accurate estimation to be made for the minimum size of polyadenylated RNA which carried functional primer. Relative to the gel mobilities of 35S

RNA (10,000 nucleotides), 28S RNA (5,500 nucleotides), 18S RNA (3,500 nucleotides), and 5S RNA (120 nucleotides), the minimum size was computed to be about 7,500 nucleotides. This locates functional primer molecules at least 7,500 nucleotides from the 3' end of the M-MuLV genome.

Location of the binding site for tRNA^{Pro} on M-MuLV genome RNA. The experiments shown in Fig. 1 indicated that functional primer molecules were situated near the 5' end of the M-MuLV genome RNA. The possibility remained open for several sites on the genome RNA to which primer could bind but that only the one (or those) near the 5' end could function in the initiation of DNA synthesis. This possibility was tested by reconstructing the complex of [^{32}P]tRNA^{Pro} and ^{3}H-labeled M-MuLV 35S RNA. The reconstructed complex was freed from unbound tRNA^{Pro} by sucrose gradient sedimentation and separated into poly(A)-deficient and poly(A)-containing fractions by chromatography on poly(U)-Sepharose. Both fractions were analyzed by electrophoresis in a low-percentage acrylamide gel followed by scintillation counting of segments of the gel tracks (Fig. 2). It is apparent that all size classes of poly(A)-deficient RNA could bind to tRNA^{Pro}, whereas only approximately full-length polyadenylated RNA subunits were able to form a complex with the tRNA. Relative to the *E. coli* 30S, 23S, and 16S rRNA markers, the minimum size of polyadenylated RNA required to bind tRNA^{Pro} was again found to be about 7,500 nucleotides.

Thermal stability of the primer-genome RNA complex. The stabilities of the primer-genome complexes isolated from virions or made in vitro were compared by measurement of the T_m for dissociation of the primer. Samples of the [^{32}P]dAMP-tagged native complex and the reconstructed complex were held at various temperatures for 2 min, quickly cooled, and analyzed by electrophoresis on 10% acrylamide gels (9). As shown in Fig. 3a, undissociated complex remained at the origin, while the primer released by thermal denaturation of the complex migrated to a characteristic position in the gel. Quantitation of the complexed and free primer by counting the Cerenkov radiation from appropriate segments of the gel yielded the data shown in Fig. 3b. The T_m values for the native and reconstructed complexes differed by less than 0.5°C and were judged to be indistinguishable by this technique. At pH 7.5 in the buffer used (10 mM NaCl–10 mM Tris-hydrochloride–1 mM EDTA), the T_m was approxmately 76°C.

Analysis of the nucleotide sequence that binds tRNA^{Pro} to the M-MuLV genome. The

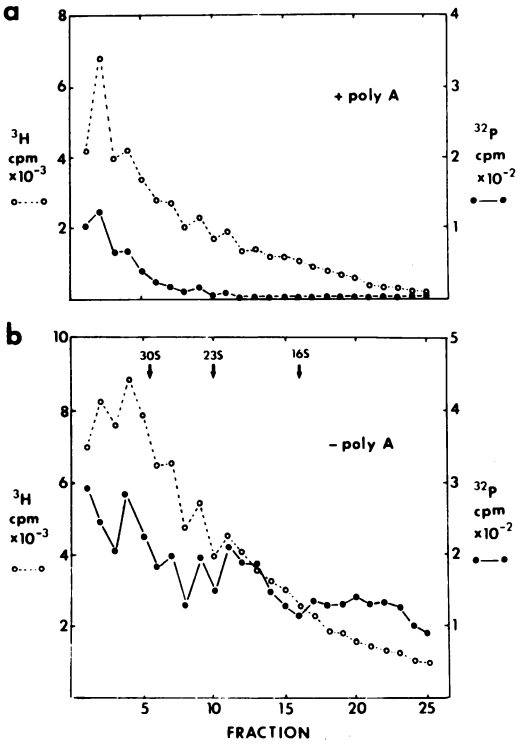

FIG. 2. *Location of the binding site for tRNA^{Pro} on the M-MuLV genome. Purified tRNA^{Pro}, uniformly labeled with ^{32}P, was annealed to ^{3}H-labeled genome RNA, and the complexes were fractionated into poly(A)-containing and poly(A)-deficient components before polyacrylamide gel electrophoresis. The distribution of ^{3}H and ^{32}P radioactivity in the gel was determined by counting 0.5-cm segments from each gel tract. E. coli 30S, 23S, and 16S rRNA's were included as size markers as indicated by the arrows. (a) Poly(A)-containing genome RNA; (b) poly(A)-deficient genome RNA.*

tRNA^{Pro} primer appeared to interact with the M-MuLV genome RNA via a region of nucleotide sequence complementarity sufficient to give a relatively high T_m. To determine the extent of this sequence, the duplex RNA formed between primer and template was isolated by virtue of its resistance to hydrolysis by RNases. The complex reconstructed from purified [^{32}P]tRNA^{Pro} and unlabeled M-MuLV genome RNA, either with or without an intermediate purification step to remove excess primer, was digested with a combination of RNases at 25°C in 0.3 M NaCl. The RNase-resistant products were analyzed by electrophoresis in polyacrylamide gels. Figure 4 shows the results of such an experiment in which pancreatic RNase was used to digest the complex. In other experiments, combinations of T_1 and pancreatic RNase, sometimes followed by

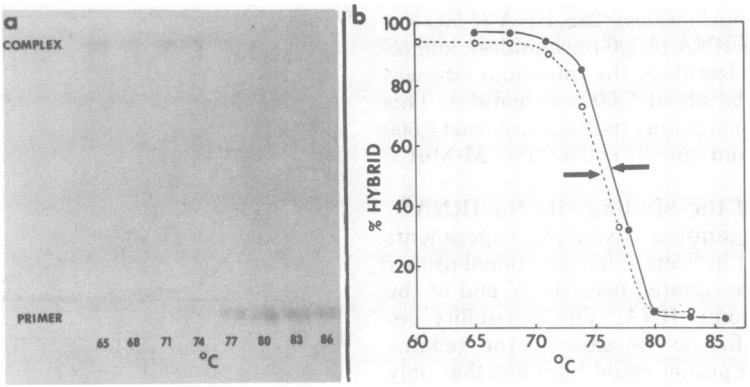

FIG. 3. *Thermal stability of the primer-genome complex in M-MuLV. Native and reconstructed complexes of ^{32}P-labeled primer and unlabeled genome RNA from M-MuLV were prepared and purified as described in the text. Samples were held at various temperatures for 2 min, quickly cooled, and analyzed by electrophoresis in 10% acrylamide gels at 400 V and 15°C for 3 h (9). The samples were loaded onto the gels in 1% agarose at 50 to 60°C so that the high-molecular-weight complex remained trapped at the origin while primer molecules released by thermal denaturation migrated to a characteristic position in the gel. The ^{32}P-labeled free or complexed primers were detected by autoradiography (a). To quantitate the extent of dissociation of the primer-genome complex, the appropriate segments of the gel were cut out and counted for Cerenkov radiation, yielding the data shown in (b). The arrows depict the T_m value, i.e., the temperature at which 50% dissociation has occurred for either the native complex tagged with [^{32}P]dAMP (●) or the reconstructed complex of [^{32}P]tRNA^{Pro} and M-MuLV viral RNA* (○).

RNase T_2, were found to give analogous results. Occasionally, multiple (up to four) bands of RNase-resistant material were detected, but further analyses showed that these probably contained a unique fragment of [^{32}P]RNA from the primer annealed to heterogeneous pieces of unlabeled genome RNA. The RNase-resistant fragment(s) was not detected in controls in which genome RNA was omitted (track labeled as "minus" in Fig. 4) or in which primer and template were mixed but not annealed.

The fragments of duplex RNA recovered from the gels were subjected to conventional nucleotide sequence analysis by RNase fingerprinting under conditions in which secondary structure was destabilized (no salt, 37°C) (1, 2). Both the T_1 (Fig. 5a) and pancreatic (Fig. 5b) RNase fingerprints of the protected fragment were less complicated than those obtained from complete tRNA^{Pro} (Fig. 5c and d). With T_1 RNase (Fig. 5a), a number of oligonucleotides were observed which did not have counterparts in the fingerprint of complete tRNA^{Pro} (Fig. 5c). All of the numbered oligonucleotides were eluted and further analyzed by established procedures as summarized in Table 1 (1, 2, 24).

Oligonucleotides 2, 3, and 5, including the cyclic phosphate forms 3! and 5!, were obtained in equimolar yields and were most likely derived from the amino acid acceptor stem of the tRNA^{Pro} (Fig. 6). Further analysis of oligonucleotide 7 by digestion with pancreatic RNase, RNase T_2, or snake venom phosphodiesterase showed that it was $(C_p)_n$, consistent with its being derived from the 3′ terminus of tRNA^{Pro} (Fig. 6). This was confirmed by coelectrophoresis of oligonucleotide 7 with the 3′-terminal T_1 oligonucleotide from tRNA^{Pro} from which the terminal adenosine had been chemically removed by periodate oxidation and β-elimination (22). In addition, partial alkaline hydrolysis and paper electrophoresis revealed five products corresponding to $(C_p)_5$ and its partial degradation products.

Further analysis of oligonucleotides 8 and 9, which did not have counterparts in the fingerprint of tRNA^{Pro}, showed that they were closely related and represented cyclic and noncyclic forms of the sequence A-m^{1}A-A-U-C-C-C-Gp. However, in experiments in which the digestion of the primer-template complex included treatment with RNase T_2, oligonucleotide 8 was contaminated with m^{1}A-A-U-C-C-C-Gp, i.e., oligonucleotide 9 from which the 5′ A- had been removed. The level of this shortened product never exceeded 25% of the total material in oligonucleotides 8 and 9.

Oligonucleotide 10 was obtained in variable yields. This product was the same as oligonucleotide 9 except for the presence of m^{6}A instead of m^{1}A. Exposure of the RNA to the mildly alkaline conditions of the gel electrophoresis buffer was probably sufficient to cause a variable conversion of m^{1}A to m^{6}A, accounting for the appear-

ance of oligonucleotide 10.

Taken together, the results from the RNase T_1 fingerprints and oligonucleotide analysis implied that a contiguous sequence of 19 bases, extending from the penultimate nucleotide at the 3′ end of the $tRNA^{Pro}$, was involved in the interaction with the M-MuLV genome RNA (Fig. 6). Parallel analysis by fingerprinting with pancreatic RNase confirmed this conclusion but provided no additional information (Table 1). The reason for the incomplete digestion of oligonucleotides 15 and 16 is unclear but may result from incomplete destabilization of the duplex RNA structure during digestion before fingerprinting.

The results obtained by Cordell et al. (6) for the analogous interaction between $tRNA^{Trp}$ and RSV genome RNA are included for comparison in Fig. 6.

DISCUSSION

Many similarities exist between the avian leukosis-sarcoma virus and murine leukemia virus groups at the level of initiation of RNA-directed DNA synthesis. In both systems, one of the host cell tRNA's associated with the genome RNA in the 70S complex serves as the major primer for reverse transcription in vitro (10, 27). The two tRNA primers identified so far, although specific for different amino acids, tryptophan and proline, share an unusual nucleotide sequence feature in the pseudouridine loop (19; Harada et al., J. Biol. Chem., in press). The initial complementary DNA products are short, begin with the same unique sequence of six deoxynucleotides, and appear to be encoded by the 5′-terminal region of the genome RNA (11, 20, 21, 32). We have extended the comparison between the avian and murine viruses by examining several aspects of the interaction between $tRNA^{Pro}$ and the genome RNA of M-MuLV.

Functional primer molecules are bound near the 5′ end of the M-MuLV genome RNA both in the virion and after in vitro binding of purified $tRNA^{Pro}$ to the M-MuLV genome RNA. It seems likely that both the native and reconstructed complexes use the same primer binding site. The two types of complex are indistinguishable in terms of the location of the primer on the genomic RNA as measured from the 3′ end, although such measurements are inherently inaccurate since they involve resolution of very large RNA chains which may differ by only a few hundred nucleotides out of a total of 10,000. The T_m for the dissociation of both complexes is the same, namely, 76°C in low-ionic-strength buffer. In these respects, the interaction between $tRNA^{Pro}$ and M-MuLV viral RNA is entirely analogous to the equivalent situation with $tRNA^{Trp}$ and RSV RNA (30, 31, 33).

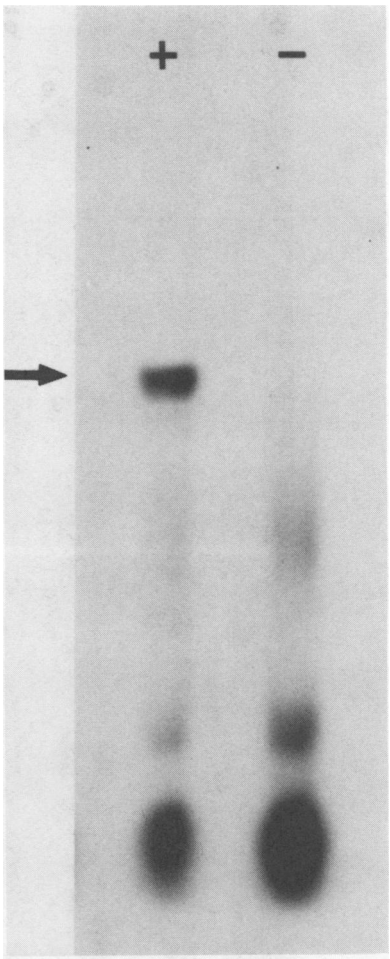

FIG. 4. *Isolation of RNase-resistant duplex formed (arrow) between $tRNA^{Pro}$ and M-MuLV genome RNA. The complex of [^{32}P]$tRNA^{Pro}$ annealed to M-MuLV genome RNA was prepared as described in the text. This complex, corresponding to a total of about 50 μg of RNA plus an additional 50 μg of carrier RNA, was digested with 5 μg of pancreatic RNase, in a total volume of 30 μl of 0.3 M NaCl-0.01 M Tris-hydrochloride (pH 7.5)-0.01 M EDTA for 1 h at 25°C. The products were analyzed directly by electrophoresis in a 20% acrylamide gel at 400 V and 15°C for 5 h (23) and detected by autoradiography. The "+" channel refers to the annealed $tRNA^{Pro}$-35S RNA complex, and the "−" channel depicts a control in which the genome RNA was omitted.*

Since the reconstructed complex of [^{32}P]-$tRNA^{Pro}$ and M-MuLV 35S RNA has properties similar to the native complex, we used the reconstructed form to identify the nucleotide sequence by which the primer bound to the genome RNA. A contiguous sequence of 19 nucleo-

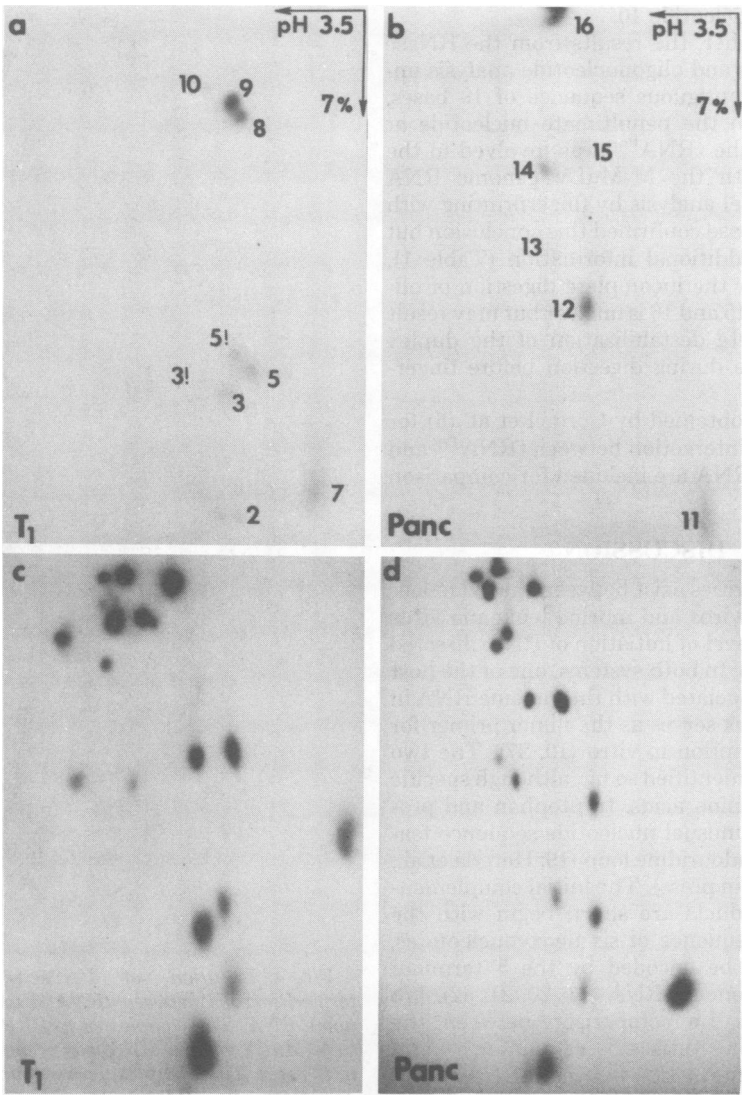

FIG. 5. *Fingerprint analysis of the nucleotide sequence which binds $tRNA^{Pro}$ to M-MuLV genome RNA. The RNase-resistant duplex formed between $tRNA^{Pro}$ and M-MuLV genome RNA was isolated as shown in Fig. 4, except that an additional digestion with RNase T_2 was included (see text). The protected fragment was eluted from the gel and analyzed by T_1 and pancreatic RNase fingerprinting (1, 2). Only oligonucleotides derived from the [^{32}P]$tRNA^{Pro}$ were detected by autoradiography. (a) RNase T_1 fingerprint of protected sequence. (b) Pancreatic RNase fingerprint of protected sequence. (c) RNase T_1 fingerprint of complete $tRNA^{Pro}$. (d) Pancreatic RNase fingerprint of $tRNA^{Pro}$. 3! and 5! refer to 2',3'-cyclic phosphate forms of oligonucleotides 3 and 5.*

tides extending from the penultimate base at the 3′ end of the $tRNA^{Pro}$ was protected against nuclease digestion as a result of interaction with the M-MuLV genome RNA. This is compared in Fig. 6 with the sequence of 16 nucleotides by which $tRNA^{Trp}$ binds to RSV viral RNA (6, 13). Although there are no obvious common features in the two sequences, they both have a high guanine-plus-cytosine content of 70%.

The sequence in $tRNA^{Pro}$ which interacts with the M-MuLV genome is depicted in Fig. 6 with ambiguity at the 5′ end since the A-m^1A bond was shown to be partially sensitive to cleavage by RNase T_2 (Table 1). However, cleavage between these two nucleotides never exceeded 25% although this bond is normally totally sensitive

TABLE 1. *Nucleotide analysis of sequences in tRNA^{Pro} which bind to M-MuLV genome RNA*[a]

Oligonucleotide	Deduced sequence	Analyses performed
2	Gp	a, c
3	A-Gp	a, c
5	A-C-Gp	a, c
7	C-C-C-C-Cp	a, c, d, e
8	A-m^{1}A-A-U-C-C-C-Gp + m^{1}A-A-U-C-C-C-Gp	a, b, c
9	A-m^{1}A-A-U-C-C-C-Gp	a, b, c
10	A-m^{6}A-A-U-C-C-C-Gp	a, b, c
11	Cp	c
12	A-m^{1}A-A-Up	c
13	A-m^{6}A-A-Up	c
14	G-G-A-Cp + G-A-G-Cp	c
15	G-A-G-C-Cp	c
16	Undigested	c

[a] The oligonucleotides derived from fingerprinting the duplex formed between tRNA^{Pro} and M-MuLV RNA, numbered as in Fig. 5, were eluted and characterized by established RNA sequencing techniques (1, 2, 24). In some cases, the complete sequence was not unambiguously deduced but was inferred from comparison of the partial analyses with the known sequence of tRNA^{Pro} (Fig. 6; Harada et al., J. Biol. Chem., in press). Analyses performed were as follows: (a) redigestion with pancreatic RNase and electrophoresis on DEAE-cellulose paper at pH 3.5; (b) redigestion with RNase U_2 and electrophoresis on DEAE-cellulose paper at pH 3.5; (c) redigestion with RNase T_2 and two-dimensional thin-layer chromatography; (d) complete digestion with snake venom phosphodiesterase and two-dimensional thin-layer chromatography; (e) partial hydrolysis with alkali and electrophoresis on DEAE-cellulose paper at pH 3.5.

to hydrolysis by RNase T_2. Thus, it is likely that the the 5'-A residue is indeed base paired to the 35S RNA.

The 3'-terminal A residue of tRNA^{Pro} was not protected against nuclease digestion even though the template RNA contained a complementary -U-G- sequence (4). A similar situation has been reported for the interaction between tRNA^{Trp} and RSV RNA (5, 6, 13). The reason for this lack of protection is unclear: it may result from unusual structures at the DNA synthesis initiation sites.

tRNA^{Pro} and tRNA^{Trp} are the only tRNA's, for which nucleotide sequence information is available, that contain the unusual sequence -G-ψ-ψ-C- in the pseudouridine loop (19; Harada et al., J. Biol. Chem., in press). The significance of this feature remains uncertain since it does not appear to be involved in the interaction between the tRNA primer and the genome RNA as assayed in these experiments. It may, however, be responsible for recognition of the primer by the viral DNA polymerase (22, 25) or for binding of the polymerase to the primer-template complex (7). It remains to be seen whether

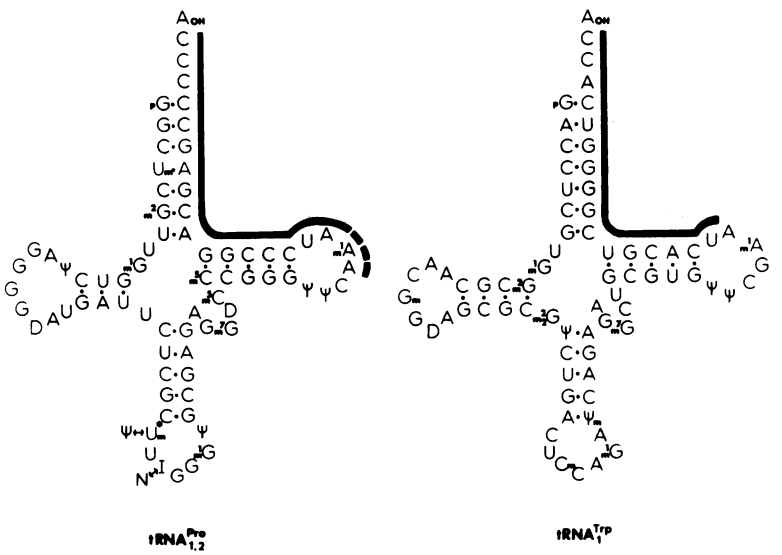

FIG. 6. *Nucleotide sequences from tRNA^{Pro} and tRNA^{Trp} responsible for binding to M-MuLV and RSV genome RNA, respectively. The complete sequences of tRNA^{Pro} and tRNA^{Trp} are presented in the cloverleaf form (19; Harada et al., J. Biol. Chem., in press). The solid line on each structure depicts the nucleotides involved in the interaction with the respective genome RNAs. The information for the binding of tRNA^{Trp} to RSV genome RNA is from Cordell et al. (6) and our unpublished data.*

this and the many other parallels that can be drawn between M-MuLV and RSV can be extended to include other members of the retrovirus group.

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