Interaction of Tryptophan tRNA and Avian Myeloblastosis Virus Reverse Transcriptase: Further Characterization of the Binding Reaction†

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ABSTRACT: The interaction between the RNA tumor virus reverse transcriptase and the tRNA primers that initiate DNA synthesis has been studied. The binding of $tRNA^{Trp}$ by avian myeloblastosis virus (AMV) reverse transcriptase is not strongly influenced by ionic strength or the presence of divalent cations. The enzyme will not bind fragments of $tRNA^{Trp}$ produced by cleavage at the anticodon loop or at the m^7G residue suggesting that much of the tRNA structure is required for effective binding. However, $tRNA^{Trp}$ modified in various ways at its 3'-OH end is bound, indicating that that end of the molecule is not crucial to the binding. The α subunit of the AMV reverse transcriptase does not detectably bind $tRNA^{Trp}$ nor does the subunit transcribe AMV 70S RNA efficiently. The AMV holoenzyme efficiently transcribes both Rous sarcoma virus (RSV) and Moloney murine leukemia virus (M-MuLV) 70S RNAs. $tRNA^{Trp}$ is the primer used when the AMV enzyme transcribes the RSV template, while $tRNA^{Pro}$ is the primer used when the AMV enzyme transcribes the M-MuLV template. The M-MuLV reverse transcriptase copies M-MuLV, AMV, and RSV 70S RNAs efficiently. However, M-MuLV reverse transcriptase does not tightly bind $tRNA^{Pro}$ or $tRNA^{Trp}$.

The virions of the RNA tumor viruses contain a DNA polymerase (reverse transcriptase) which can copy the genome RNA into complementary DNA using a low-molecular-weight RNA as primer (Temin and Baltimore, 1972). For Rous sarcoma virus (RSV), the primer RNA has been shown to be cellular tryptophan-tRNA ($tRNA^{Trp}$) (Harada et al., 1975). Cellular $tRNA^{Trp}$ has two binding specificities that allow it to function as a primer: it hybridizes to a site on 35S RNA (Eiden et al., 1976; Cordell et al., 1976) and it binds specifically to the reverse transcriptase (Panet et al., 1975).

In this communication we examine the interaction between $tRNA^{Trp}$ and avian myeloblastosis virus (AMV) reverse transcriptase in more detail. We have studied the ability of one of the two subunits of the enzyme to bind $tRNA^{Trp}$, the effect of inhibitors on the reaction, and the ability of the enzyme to bind modified $tRNA^{Trp}$. The interaction of both avian and murine reverse transcriptases with $tRNA^{Pro}$, the primer in murine viral 70S RNA (Peters et al., 1977), was also studied.

Experimental Section

Purification of the AMV and Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase. AMV reverse transcriptase was purified to homogeneity by chromatography on DNA-cellulose from a preparation supplied by Dr. Joseph Beard (Life Sciences, Inc., St. Petersburg, Fla.). The enzyme (6×10^5 units as defined by Panet et al., 1975) was dialyzed against buffer A (50 mM Tris,[1] pH 8.0, 0.1% Nonidet P-40, 10 mM mercaptoethanol, 20% glycerol, and 0.1 mM EDTA) and loaded onto a DNA-cellulose column (8×55 mm) prepared by the method of Bautz and Dunn (1972). It was eluted from the column with a 50-mL gradient of 0.0–0.6 M KCl in buffer A and the enzymatic activity of each fraction was determined by incorporation of dGMP with poly(C)·oligo(dG) as template·primer. Eight percent of the activity was present in the void volume of the column, 25% eluted from the column at 0.05 M KCl, and the rest eluted at 0.24 M KCl. Analysis of the fractions by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate showed that the fraction eluting from the column in the void volume and at 0.05 M KCl was highly enriched for the α subunit. The activity which eluted at 0.24 M KCl was stored frozen at −70 °C and used directly as holoenzyme. The fractions eluting at 0.05 M KCl were pooled, dialyzed against buffer A for 4 h, and loaded onto a phosphocellulose column. The enzyme was eluted from the column in a concentrated form by washing with buffer A containing 0.4 M NaCl. After adding glycerol to a final concentration of 50%, the enzyme fraction was stored at −70 °C. Electrophoresis of this preparation of α subunit showed less than 2.5% of β subunit.

Moloney MuLV reverse transcriptase was purified from 80 mg of detergent-disrupted virions by sequential chromatography on columns of DEAE-Sephadex and phosphocellulose. Greater than 90% of protein migrated as a single band of molecular weight 85 000 on sodium dodecyl sulfate-polyacrylamide gels.

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; DME, Dulbecco's modified Eagle's medium; TSE buffer, 0.02 M Tris-HCl (pH 7.8)–0.1 M NaCl–0.001 M EDTA; TE buffer, 0.045 M Tris base–0.045 M boric acid–0.0014 M EDTA.

Preparation of ^{32}P-Labeled RNAs. Cultures of chicken embryo fibroblasts infected with the Schmidt-Ruppin D strain of Rous sarcoma virus were labeled with ^{32}P as described previously (Sawyer and Dahlberg, 1973; Panet et al., 1975). The viral and cellular 4S RNA fractions were prepared as described by Panet et al. (1975).

For purification of large amounts of ^{32}P-labeled $tRNA^{Trp}$, uninfected chicken embryo fibroblast cultures were labeled with $^{32}PO_4$, as above. After removal of the medium, the cells were treated on the culture plate (1 mL per 100-mm dish) at room temperature for 30 min with TSE buffer (0.02 M Tris-HCl (pH 7.8)–0.1 M NaCl–0.001 M EDTA) containing 1% Triton X-100. The cell material was gently scraped from the plate with a rubber spatula and the suspension centrifuged at 750*g* for 10 min (Sorvall HB4, 2500 rpm) to deposit nuclei and debris. The supernatant was then extracted with phenol and chloroform–isoamyl alcohol (24:1) and the nucleic acids were precipitated with 2 volumes of ethanol. The cytoplasmic RNA was then separated into 28S, 18S, and 4S fractions by centrifugation through 18-mL gradients of 5–20% sucrose in TSE + 0.5% Sarkosyl for 18 h at 5 °C, 26 000 rpm in a Beckman SW27 rotor.

The individual tRNA species were purified from virion-associated or cellular tRNA using two-dimensional gel electrophoresis as described previously (Ikemura and Dahlberg, 1973).

^{32}P-labeled Moloney MuLV was prepared from a 3T3 producer line. The cells in roller bottles were grown in Dulbecco's modified Eagle's (DME) medium which contained 10% calf serum. Before labeling, the cells were washed twice with phosphate-free DME. They were incubated 4–8 h with phosphate-free DME and 10% calf serum which was dialyzed against 0.1 M NaCl to remove phosphate. The cells were again washed with phosphate-free DME and 30 mL of phosphate-free DME containing 10% dialyzed calf serum and 20 mCi of carrier-free ^{32}P-labeled phosphoric acid (New England Nuclear Corp., Boston, Mass.) was added. The cells were exposed to ^{32}P for 12 h. The medium was then collected and 25 mL of DME containing 10% calf serum was added. Supernatants containing the virus were collected after 12, 24, and 36 h. The virions were purified from the pooled supernatants as described by Fan and Baltimore (1973). Virion RNAs were purified as described above for ^{32}P-labeled RSV. ^{32}P-labeled cellular RNAs were purified from the radioactively labeled 3T3 cells as described above for chick cells.

Chicken liver tRNA and crude preparations of chicken liver aminoacyl tRNA synthetases were purified as described previously (Rogg et al., 1969; Nishimura and Weinstein, 1969; Weinstein et al., 1966). Charging of tRNA with [^3H]tryptophan (11 Ci/mmol), [^{35}S]methionine (236 Ci/mmol), or [^3H]lysine (50 Ci/mmol) was carried out as described by Nishimura and Weinstein (1969). Radioactive nucleotides and amino acids were purchased from New England Nuclear Corp., Boston, Mass. Monospecific antiserum to AMV DNA polymerase was obtained from Dr. R. Nowinski (Nowinski et al., 1972) and the IgG fraction was purified from the serum by chromatography on Sephadex G-200 column (Panet et al., 1974).

Modification of $tRNA^{Trp}$. (a) S_1 Cleavage. ^{32}P-labeled $tRNA^{Trp}$ was treated with nuclease S_1 as described by Harada and Dahlberg (1975). Under appropriate conditions, specific cleavage occurs in the anticodon loop and in the CCA-OH at the 3′ terminus. The 5′ and 3′ "halves" of the molecule were isolated by two-dimensional gel electrophoresis as described previously (Harada and Dahlberg, 1975).

(b) m^7G Cleavage. $tRNA^{Trp}$ contains m^7G located 31 residues from the 3′ terminus. Cleavage at m^7G was carried out according to the procedure of Wintermeyer and Zachau (1970). The 5′ and 3′ fragments were purified by gel electrophoresis (Harada et al., 1975).

(c) Periodate Oxidation and β Elimination. The 3′-OH end of $tRNA^{Trp}$ was modified by periodate oxidation and reduction essentially as described by Fraenkel-Conrat and Steinschneider (1968). $tRNA^{Trp}$ was oxidized by the addition of 5 μL of fresh 0.1 M sodium periodate to 100 μL of 0.1 M sodium acetate (pH 6.0) containing the RNA and 75 μg of carrier RNA. After keeping the mixture at room temperature for 1 h in the dark, 10 μL of 80% glycerol was added to destroy excess periodate and the products were recovered by ethanol precipitation. One-third of the treated RNA was retained as periodate oxidized $tRNA^{Trp}$; the remainder was dissolved in 0.04 mL of 0.1 M sodium acetate (pH 5.0) to which 0.16 mL of 0.3 M aniline hydrochloride (pH 5.0) was added. After keeping the mixture 1 h at room temperature in the dark, the products were again recovered by ethanol precipitation. One-half of this β-eliminated material was retained, while the remainder was treated with 5 mg/mL alkaline phosphatase in 0.1 M Tris-HCl (pH 8.4) at 37 °C for 1 h. The phosphatase was removed by phenol extraction, ether extraction, and ethanol precipitation. The resultant four samples of $tRNA^{Trp}$—(a) untreated $tRNA^{Trp}$, (b) periodate oxidized $tRNA^{Trp}$, (c) β-eliminated $tRNA^{Trp}$, and (d) β-eliminated phosphatase-treated $tRNA^{Trp}$ each containing approximately 25 μg of carrier RNA and equivalent amounts of ^{32}P-labeled $tRNA^{Trp}$—were ethanol precipitated twice more and used directly in the binding assays. Samples of the various cleavage products and modified $tRNA^{Trp}$ preparation were analyzed by ribonuclease T_1 fingerprinting (Sanger et al., 1965) before use in the binding assays.

Binding Assays. (a) Gel Filtration. Reaction mixtures (100–200 μL) contained 50 mM Tris-HCl (pH 8.3), 60 mM NaCl, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 5% glycerol, 0.1% Nonidet P-40, 50–150 enzyme units of reverse transcriptase, and the amount of RNA indicated. After 10 min of incubation at 0 °C, the reaction mixtures were chromatographed on Sephadex G-100 (0.6 × 23 cm) preequilibrated at 4 °C with a buffer containing 0.1 M potassium phosphate, pH 7.5, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10% glycerol, 0.1 mM EDTA, and 0.1% Nonidet P-40. The columns were washed with this buffer. Fractions of 0.17 mL were collected and 25–30-μL portions of each fraction were assayed for reverse transcriptase activity; 60–70% of added enzymatic activity was routinely recovered. Portions of each of the fractions were also assayed for radioactive RNA which was recovered in 70–80% yield.

To obtain the percent of input RNA that was bound by the reverse transcriptase, the percent of the input RNA which eluted in the void volume of the G-100 column in the absence of enzyme was subtracted from the percent that eluted in the void volume in the presence of the enzyme. The background for this assay was routinely less than 2%.

(b) Glycerol Gradient Centrifugation. Reaction mixtures of 100 μL were prepared as in gel filtration analysis. The mixtures were layered onto 5 mL of 20–40% glycerol gradients in 0.1 M potassium phosphate (pH 7.5), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1% Nonidet P-40, and 0.1 mM EDTA. After centrifugation for 24 h at 49 000 rpm at 7 °C in an SW 50.1 Beckman rotor, the gradients were fractionated into 0.175-mL aliquots and assayed for reverse transcriptase.

Preparation of the Elongated Primer. Purified RSV at a final concentration of 5 mg/mL was incubated for 30 min at 37 °C in a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 50 mM NaCl, 6 mM $MgCl_2$, 0.02% Nonidet P-40, 5 mM

TABLE I: The Effect of Reaction Conditions on the Binding of $tRNA^{Trp}$.[a]

Reaction and elution conditions	% of $tRNA^{Trp}$ in excluded vol	% of AMV reverse transcriptase act. recovered
Complete	69	78
− Mg^{2+}, + 1 mM EDTA	82	74
− Nonidet P-40	<1	<1
− PO_4^{2-}, + Tris 50 mM (pH 7.5)	16	3
− PO_4^{2-}, + Tris 50 mM (pH 8.3)	18	4
+ 0.5 M KCl	67	75
− β-mercaptoethanol	5	2.5
− glycerol	<1	<1
Complete, 25 °C	70	75

[a] The complete reaction mixture (100 μL) contained 50 mM Tris-HCl (pH 8.3), 50 mM NaCl, 0.1% Nonidet P-40, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10% glycerol. The standard elution buffer contained 100 mM NaH_2PO_4 (pH 7.5), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10% glycerol, 0.1% Nonidet P-40. The AMV enzyme (1 μg) was added last to each reaction. The reaction mixtures were incubated at 0 °C for 10 min before loading onto the columns which were run at 4 °C. The changes in the reaction conditions indicated above were made in both the reaction mixtures and the elution buffer. Each reaction contained 1×10^4 cpm of $tRNA^{Trp}$ (specific activity about 1×10^6 cpm/μg) purified from cultured chick embryo fibroblasts.

dithiothreitol, 1 mM dGTP, 1 mM TTP (P-L Biochemicals), and 4 μM [α-^{32}P]dATP, specific activity 100 Ci/mmol (New England Nuclear Corp.), 0.03 M creatine phosphate, and 0.01 mg/mL creatine phosphokinase. Reactions were terminated by adding 2 mL of a buffer containing 0.01 M Tris-HCl (pH 7.5), 10 mM EDTA, and 0.2 M sodium acetate (stopping buffer) and extracted twice with 4 mL of phenol–chloroform (1:1). The aqueous phases were pooled and the nucleic acids precipitated with 2 volumes of ethanol after addition of 20 μg of yeast RNA as carrier. The precipitates were collected by centrifugation for 5 h at 0 °C at 35 000 rpm in a Beckman SW 41 rotor. The pellets were lyophilized and resuspended in 500 μL of the stopping buffer containing 0.1% sodium dodecyl sulfate. The nucleic acid was reprecipitated by addition of 2 volumes of ethanol and centrifugation 30 min in a Brinkman 3200 centrifuge. The pellet was lyophilized and resuspended in TE buffer (0.045 M Tris base, 0.045 M boric acid, and 0.0014 M EDTA) which contained 0.1% sodium dodecyl sulfate and 10% glycerol, and applied to a 10% polyacrylamide slab gel. Electrophoresis was for 2 h at 400 V and 0 °C in an EC slab gel apparatus. The gels were autoradiographed and the product corresponding to the tRNA with limited number of added deoxynucleotides was eluted from the crushed gel fragments in 0.2 M NaCl–0.01 M Tris-HCl (pH 7.5)–1 mM EDTA with 20 μg of yeast RNA as carrier. The nucleic acids were reprecipitated as described above, lyophilized, and resuspended in 0.01 M Tris-HCl (pH 8.3)–1 mM EDTA.

A fraction of the tRNA primer elongated with DNA was treated with 10% piperidine overnight at 37 °C to hydrolyze the RNA, lyophilized, and resuspended in water. The resulting DNA fragment was used in a gradient binding assay. The sequence of the nucleotide fragment was found to be AATGAAG (Haseltine et al., 1976) in agreement with the nucleotide sequence determined by Taylor et al. (1975).

Enzymatic Assays. Reaction mixtures for reverse transcription (100 μL) contained 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 0.1 mM [^{3}H]dGTP (100 cpm/pmol), 2 μg of poly(C), and 0.5 μg of oligo(dG)$_{12-18}$ (Collaborative Research, Waltham, Mass.), 5 mM dithiothreitol, and 10–50 μL of the column or gradient fractions. Reactions were incubated for 30 min at 37 °C. One enzyme unit was defined as the amount needed to catalyze the incorporation of 1 pmol of dGMP into polymer in 1 min under these conditions. The assay for *E. coli* polymerase I was as described for reverse transcriptase but 1 μg of poly(dA) and 1 μg of oligo(dT)$_{12-18}$ were used as the template and primer and the incorporation of [^{3}H]dTTP (60 cpm/pmol) was followed.

Results

In previous work, we showed by both Sephadex G-100 chromatography and glycerol gradient centrifugation that AMV reverse transcriptase can bind $tRNA^{Trp}$ (Panet et al., 1975). To further investigate the requirements of the binding reaction, the components of the reaction mixture were varied and the effect on binding was measured by Sephadex G-100 chromatography. The standard reaction conditions involved incubation of the tRNA and enzyme at 0 °C in the presence of 10 mM $MgCl_2$ and 60 mM NaCl followed by gel filtration in a buffer containing 0.1 M sodium phosphate (pH 7.5), 0.1% Nonidet P-40, 10-mM β-mercaptoethanol, 10% glycerol, and 5 mM $MgCl_2$. The β-mercaptoethanol, Nonidet P-40, and glycerol were all required for the recovery of enzymatic activity (Table I). The stability of the enzyme was also adversely affected if 50 mM Tris-HCl (pH 8.3 or 7.5) was used instead of 0.1 M sodium phosphate in the elution buffer. AMV reverse transcriptase bound $tRNA^{Trp}$ even if the salt concentration of the reaction and the elution buffer was raised to 0.5 M KCl. The fraction of $tRNA^{Trp}$ bound was slightly increased when the magnesium in both the reaction mixture and the column elution buffer was replaced by 1 mM EDTA (Table I, line 2; also see Grandgenett et al., 1976). Increasing the temperature of incubation for the binding reaction and assay to 25 °C did not affect the amount of $tRNA^{Trp}$ bound.

Inhibition of Binding. Two inhibitors of the DNA polymerase activity of reverse transcriptase were tested for their ability to inhibit the binding of $tRNA^{Trp}$ to the enzyme: an antibody against reverse transcriptase and *N*-ethylmaleimide. In the absence of inhibitors, binding was evident as [^{32}P]$tRNA^{Trp}$ eluting in the void volume of the Sephadex column (Figure 1A). Anti-reverse transcriptase IgG prepared from the serum of a rat immunized with pure AMV reverse transcriptase inhibited both the DNA polymerase activity (Panet et al., 1974) and the $tRNA^{Trp}$ binding activity (Figure 1B). Non-specific rat IgG inhibited neither the DNA polymerase activity nor the $tRNA^{Trp}$ binding (data not shown). *N*-Ethylmaleimide, a reagent that reacts with cysteinyl groups of the enzyme and is a nonreversible inhibitor of the reverse transcriptase (Panet and Baltimore, unpublished results), inhibited the ability of the enzyme to bind $tRNA^{Trp}$ (Figure 1C).

Displacement of $tRNA^{Trp}$. To determine whether the binding of $tRNA^{Trp}$ by AMV holoenzyme is a reversible reaction, we tested the ability of unlabeled $tRNA^{Trp}$ and a mixture of unfractionated tRNA to displace [^{32}P]$tRNA^{Trp}$ from a tRNA–enzyme complex. A 10-fold molar excess of $tRNA^{Trp}$ to enzyme protein or a 200-fold excess of unfractionated chick cell tRNA displaced [^{32}P]tRNA from a preformed tRNA–enzyme complex (Table II, lines 2 and 3). Both $tRNA^{Trp}$ and unfractionated chick cell tRNA also prevented the formation of a [^{32}P]$tRNA^{Trp}$–enzyme complex when added in sufficient molar excess (Table II). Purified $tRNA^{Trp}$ competed 10 to 15 times more efficiently for binding of [^{32}P]$tRNA^{Trp}$ than did unfractionated chick cell tRNA. This

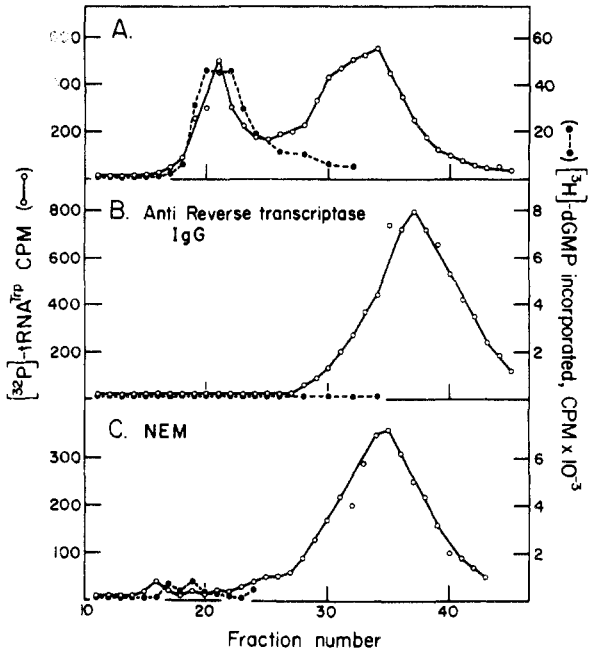

FIGURE 1: Inhibition of DNA polymerase and $tRNA^{Trp}$ binding activities by monospecific antibody and *N*-ethylmaleimide. (A and B) The reactions were conducted in two steps: reverse transcriptase (50 units) was preincubated with (B) or without (A) anti-AMV reverse transcriptase IgG (20 μL) in a total volume of 100 μL with buffer B (10 mM Tris-HCl, pH 8.0, 100 mM KCl, and 0.5 mg of bovine serum albumin per mL). After 15 min at room temperature, the reactions were transferred to 4 °C. Cell [^{32}P]$tRNA^{Trp}$ (12 000 cpm) was added together with the other components of the binding reaction and the reactions were analyzed by chromatography on Sephadex G-100 columns. (C) Reverse transcriptase (50 units) was preincubated with 3 mM *N*-ethylmaleimide (NEM) in a total volume of 100 μL with buffer B. After 15 min at 4 °C, dithiothreitol (20 mM) was added to destroy excess of NEM. Cell [^{32}P]$tRNA^{Trp}$ (6000 cpm) and the rest of the components for the binding assayed were added as described in the text. (o—o) [^{32}P]$tRNA^{Trp}$ and (•—•) reverse transcriptase.

suggested that the majority of tRNA species are unable to compete for $tRNA^{Trp}$ binding.

Binding to Modified $tRNA^{Trp}$. In an attempt to determine what part of the $tRNA^{Trp}$ binds to the enzyme, we tested the ability of AMV reverse transcriptase to bind fragments of $tRNA^{Trp}$ produced by enzymatic digestion or chemical treatments (Table III). Samples examined included $tRNA^{Trp}$ that was modified at the 3′ terminus and fragments of the RNA derived from the 5′ or 3′ regions by chemical cleavage at the m^7G or S_1 nuclease digestion at the anticodon. The RNA fragments were separated by electrophoresis in two-dimensional polyacrylamide gels. Modification of the 3′-terminal adenosine had no effect on binding to reverse transcriptase. None of the 3′ or 5′ fragments bound to the enzyme, however, suggesting that the three-dimensional architecture of the tRNA may be needed for binding.

To determine whether the reverse transcriptase can bind to aminoacylated tRNA, unfractionated chick cell tRNA was charged with either [^{3}H]tryptophan or [^{35}S]methionine and used in an enzyme binding assay using the Sephadex G-100 procedure (Table IV). We have previously shown that AMV DNA polymerase selectively binds to $tRNA^{Trp}$ and $tRNA^{Met}_4$ among an unfractionated population of small RNAs from chick cells or RSV virions (Panet et al., 1975). The AMV reverse transcriptase bound to tRNA which had been charged with tryptophan or methionine, but not to tRNA charged with lysine or phenylalanine. The fact that a rather low fraction of the input aminoacylated tRNA eluted with the

TABLE II: Displacement and Competition of $tRNA^{Trp}$ from AMV Reverse Transcriptase.[a]

Competitor added	% [^{32}P]$tRNA^{Trp}$ bound
A. Displacement	
None	54
$tRNA^{Trp}$, 2 μg	2
Unfractionated chick tRNA, 20 μg	2
B. Competition	
$tRNA^{Trp}$, 0.05 μg	41
$tRNA^{Trp}$, 0.2 μg	15
tRN&supcTrp, 1.0 μg	2
Unfractionated chick tRNA, 0.8 μg	38
Unfractionated chick tRNA, 3.0 μg	17
Unfractionated chick tRNA, 15.0 μg	3

[a] The amount of [^{32}P]tRNA bound was determined by the Sephadex G-100 assay. The amount bound is that percent of [^{32}P]tRNA added to the reaction which elutes from the column in the void volume, together with the enzyme activity. Reactions were 100 μL and contained 1 μg of AMV holoenzyme protein. For the displacement experiments (lines 1-3), reactions containing [^{32}P]$tRNA^{Trp}$ and AMV reverse transcriptase were incubated at 0 °C for 10 min. The competitor was then added and incubated for an additional 10 min at 0 °C before they were layered onto the Sephadex G-100 columns. For the reactions of lines 4-9, the competitor and enzyme were incubated for 5 min at 0 °C and then the [^{32}P]$tRNA^{Trp}$ was added. The reactions were incubated for 10 min more and then layered onto the Sephadex G-100 columns. $tRNA^{Trp}$, 1×10^4 cpm (specific activity $\sim 2 \times 10^6$ cpm/μg), was added to each reaction.

TABLE III: Binding of Modified $tRNA^{Trp}$ by AMV Reverse Transcriptase.[a]

RNA	Assay	% bound
$tRNA^{Trp}$	Glycerol gradient	56
$tRNA^{Trp}$	Sephadex G-100	61
$tRNA^{Trp}$ (periodate oxidized)	Sephadex G-100	58
$tRNA^{Trp}$ (periodate oxidized, β eliminated)	Sephadex G-100	59
$tRNA^{Trp}$ (periodate oxidized, β eliminated, phosphatase treated)	Sephadex G-100	57
(^{32}P) 3′ fragment of cleavage at m^7G	Glycerol gradient	<1
(^{32}P) 3′ fragment of cleavage at m^7G	Sephadex G-100	<1
(^{32}P) 5′ fragment of cleavage at m^7G	Glycerol gradient	<1
(^{32}P) 5′ fragment of cleavage at m^7G	Sephadex G-100	<1
(^{32}P) 3′ fragment of partial S1 cleavage	Glycerol gradient	<1
(^{32}P) 3′ fragment of partial S1 cleavage	Sephadex G-100	<1
(^{32}P) 5′ fragment of partial S1 cleavage	Glycerol gradient	<1
(^{32}P) 5′ fragment of partial S1 cleavage	Sephadex G-100	<1

[a] Binding of cell $tRNA^{Trp}$ or partial digestion products was determined by either the Sephadex G-100 or glycerol gradient technique. Reactions contained in 100 μL: 1 μg of AMV reverse transcriptase (αβ complex) and 1×10^4 cpm of RNA (specific activity 2×10^6 cpm/μg). Fragments of $tRNA^{Trp}$ were prepared as described in Experimental Section.

TABLE IV: Binding of Various RNA Species by AMV Reverse Transcriptase.[a]

RNA	Origin	% bound
[^{3}H]Lys-tRNA	Chick cell	2
[^{35}S]Met-tRNA	Chick cell	18
[^{3}H]Phe-tRNA	Chick cell	2
[^{3}H]Trp-tRNA	Chick cell	32
$tRNA^{Pro}$	M-MuLV virions	32
$tRNA^{Pro}$	M-MuLV virions	31
$tRNA^{Pro}$	NRK cell	23
$tRNA^{Pro}$	NRK cell	24
Spot 7 RNA	M-MuLV virions	1
Spot 8 RNA	M-MuLV virions	1

[a] Binding was assayed by the Sephadex G-100 method except for experiments reported in lines 6 and 8 which were assayed by the glycerol gradient technique. Reactions contained in 100 μL: 1 μg of AMV reverse transcriptase ($\alpha\beta$ complex) and 1×10^4 cpm of ^{32}P-labeled purified tRNA species (specific activity 2×10^6 cpm/μg) as indicated. The [^{32}P]tRNA species were purified as described in the Experimental Section. Reactions which contained charged tRNA species (lines 1–4) contained 2 μg of AMV reverse transcriptase ($\alpha\beta$ complex) and 0.2 μg of chicken liver tRNA charged with [^{3}H]lysine, [^{35}S]methionine, [^{3}H]phenylalanine, or [^{3}H]tryptophan as indicated. Virion RNAs designated by spot number refer to molecules characterized elsewhere (Sawyer and Dahlberg, 1973; Peters et al., 1977).

TABLE V: Binding of AMV Reverse Transcriptase to an Elongated Primer.[a]

tRNA	Assay	% bound
[^{32}P]$tRNA^{Trp}$	Glycerol gradient	61
[^{32}P]$tRNA^{Trp}$	Sephadex G-100	58
$tRNA^{Trp}$-[^{32}P]dAATGAAG	Glycerol gradient	72
$tRNA^{Trp}$-[^{32}P]dAATGAAG	Sephadex G-100	68
[^{32}P]dAATGAAG	Glycerol gradient	1
[^{32}P]dAATGAAG	Sephadex G-100	1

[a] The binding of AMV reverse transcriptase to the virion nucleic acids was determined by both the glycerol gradient assay and the Sephadex G-100 column assay. Percent bound expresses the fraction of the radioactivity that sediments at 9 S on a glycerol gradient or the fraction that elutes from a G-100 column in the void volume, together with the enzymatic activity. The elongated primer was synthesized in an endogenous reaction which contained (α-^{32}P) dGTP, dATP, and dTTP but not dCTP. The elongated primer was separated from other reaction products and unreacted $tRNA^{Trp}$ on a 10% polyacrylamide gel. dAATGAAG was prepared by alkaline hydrolysis of this product. The sequence of the DNA fragment was confirmed using the methods of Sanger et al. (1973).

enzyme in the void volume could reflect either a reduced efficiency of binding compared with the corresponding uncharged tRNA or the presence of isoaccepting tRNA species in the charged mixture which are not recognized by the enzyme.

To investigate whether reverse transcriptase could bind to tRNA that had deoxynucleotides added to its 3′-OH end, a sample of tRNA which was ^{32}P-labeled by addition of 7 deoxynucleotides was prepared by incubating virions in an endogenous reverse transcriptase reaction lacking dCTP. Taylor et al. (1975) have shown and we have confirmed that, under these conditions, the sequence dAATGAAG is added to the $tRNA^{Trp}$ (Haseltine et al., 1976). The elongated tRNA was purified from unreacted $tRNA^{Trp}$ and from other reaction products by electrophoresis on a 10% polyacrylamide gel.

TABLE VI: Comparison of Activity of $\alpha\beta$ with α on Poly(rC)·Oligo(dG) and AMV 70S RNA.[a]

Template·primer	μg of $\alpha\beta$ added	pmol of dGMP incorp	μg of α added	pmol of dGMP incorp
Poly(rC)·oligo(dG)	0.04	142	0.025	112
	0.1	306	0.05	273
	0.2	937	0.1	500
AMV 70S RNA	1	0.29	1	0.05
	1	0.44	1	0.04

[a] AMV $\alpha\beta$ and α were prepared as described in the Experimental Section. Reactions using the synthetic template·primer contained (in 100 μL) 2 μg of poly(rC) and 0.05 μg of oligo(dG)$_{14}$ and 0.05 M Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 5 mM dithiothreitol, 80 μM [^{32}P]dGTP (specific activity 50 cpm/pmol) and were incubated for 30 min at 37 °C. Reactions using the natural template contained in 100 μL: 1 μg of 70S AMV RNA, and 0.05 M Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 1 mM dATP, dCTP, dTTP, and 10 μM [^{32}P]dGTP (specific activity 4000 cpm/pmol). Reactions were incubated for 90 min at 37 °C.

Binding was assayed using both the glycerol gradient and Sephadex G-100 techniques. The elongated $tRNA^{Trp}$ did form a rapidly sedimenting complex with the reverse transcriptase whereas the free heptadeoxyribonucleotide, derived by hydrolysis of the elongated tRNA, did not bind detectably (Table V).

The α Subunit of AMV Reverse Transcriptase Does Not Bind Tightly to $tRNA^{Trp}$. The AMV reverse transcriptase is a complex between two subunits of molecular weight 60 000 (α) and 90 000 (β) (Kacian et al., 1971). The α subunit has been separately purified and contains a subset of the tryptic peptides characteristic of the larger subunit (Gibson and Verma, 1975; Rho et al., 1975). The α subunit retains the catalytic sites by which the enzyme copies RNA, copies DNA, and degrades the RNA portion of a DNA-RNA hybrid (ribonuclease H activity) (Grandgenett et al., 1973; Verma, 1975). However, a template·primer complex which protects the holoenzyme from thermal inactivation fails to protect the smaller subunit from heat inactivation, suggesting reduced binding affinity of the smaller subunit for the template·primer complex (Panet et al., 1974). Grandgenett and Rho (1975) and Grandgenett et al. (1976) report and we have confirmed that the α subunit is unable to copy 70S RNA efficiently. The ratio of activity of the α subunit on 70S RNA to the activity on a synthetic template is about fivefold lower than the ratio of activities of the holoenzyme (Table VI). These observations led us to investigate whether or not the α subunit could tightly bind $tRNA^{Trp}$. Purified α subunit was prepared by eluting a commercial preparation of AMV reverse transcriptase from DNA cellulose as described in the Experimental Section.

The glycerol gradient centrifugation assay was used in these binding experiments under conditions both of enzyme excess and of $tRNA^{Trp}$ excess. [^{125}I]IgG and *E. coli* DNA polymerase I were used as sedimentation velocity markers. The holoenzyme sedimented at 7.2 S (Figures 2A and 2E), whereas the α subunit sedimented at 5.6 S (Figures 2C and 2G). When about 0.03 μg of [^{32}P]$tRNA^{Trp}$ was added to the holoenzyme under conditions of large enzyme excess, a complex sedimenting at 9 S was formed containing ^{32}P but because of the enzyme excess little of the total enzyme present was part of the complex as has been described previously (Panet et al., 1975) (Figure 2B). No such complex was formed between the α subunit and labeled $tRNA^{Trp}$ (Figure 2D). The same number

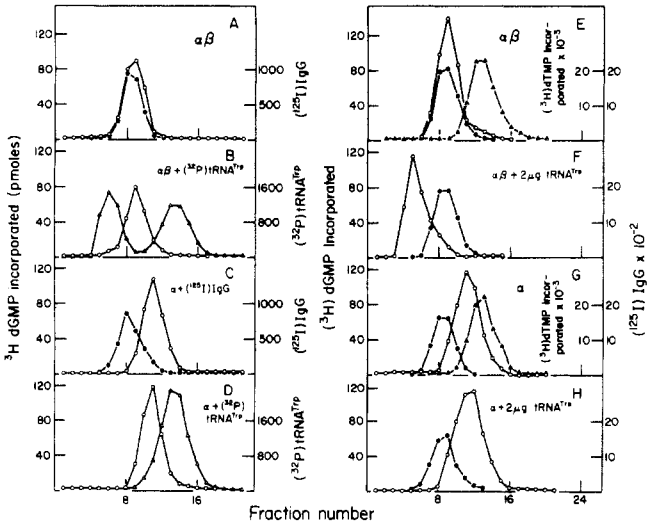

FIGURE 2: Sedimentation through glycerol gradients of mixtures of AMV reverse transcriptase with $tRNA^{Trp}$. Mixtures containing reverse transcriptase and $tRNA^{Trp}$ were layered onto 5 mL of 20–40% glycerol gradients as described in Panet et al. (1975). Fractions were assayed for $\alpha\beta$ or α reverse transcriptase activity, *E. coli* DNA polymerase I activity, and for either ^{125}I or ^{32}P. Sedimentation was from right to left. (A) $\alpha\beta$ (O—O) and [^{125}I]IgG (●—●). (B) $\alpha\beta$ (O—O) and [^{32}P]$tRNA^{Trp}$ (Δ—Δ). (C) α (O—O) and [^{125}I]IgG (●—●). (D) α (O—O) and [^{32}P]$tRNA^{Trp}$ (Δ—Δ). (E) $\alpha\beta$ (O—O) and [^{125}I]IgG (●—●) and *E. coli* polymerase I (▲—▲). (F) $\alpha\beta$ (O—O) and [^{125}I]IgG (●—●) and 2 μg of $tRNA^{Trp}$. (G) α (O—O), [^{125}I]IgG (●—●), and *E. coli* polymerase I (▲—▲). (H) α (O—O), [^{125}I]IgG (●—●), and 2 μg of $tRNA^{Trp}$.

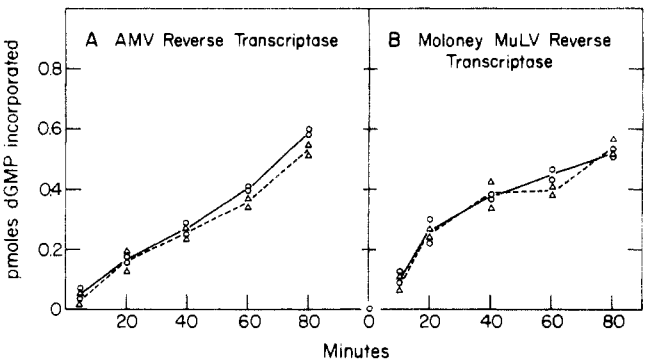

FIGURE 3: Activity of AMV and M-MuLV reverse transcriptase on AMV 70S RNA and M-MuLV 70S RNA. Reactions contained in 100 μL: 35 units of AMV reverse transcriptase (A) or 35 units of M-MuLV reverse transcriptase (B) and 2 μg of either AMV 70S RNA (O—O) or 2 μg of M-MuLV 70S RNA (Δ—Δ). The reaction conditions were 0.05 mM Tris-HCl, pH 8.3, 6 mM $MgCl_2$, 5 mM dithiothreitol, 0.02% Nonidet P-40, 60 mM KCl, and 1 mM each of dATP, dCTP, dTTP, and 20 μM [^{32}P]dGTP (specific activity 4000 cpm/pmol). Reactions were incubated at 37 °C for the times indicated.

of enzyme units of the two enzyme preparations were used, as measured by incorporation of dGMP using poly(C)·oligo(dG) as a template·primer. Mixing of the holoenzyme with a molar excess of unlabeled purified $tRNA^{Trp}$ increased the sedimentation rate of the holoenzyme from 7.2 S to 9 S (Figure 2F). The sedimentation rate of the α subunit remained unchanged when mixed with a molar excess of purified $tRNA^{Trp}$ (Figure 2H). Grandgenett et al. (1976) have also reported that the α subunit is unable to bind $tRNA^{Trp}$.

Binding to $tRNA^{Pro}$, the Primer in Moloney MuLV. Recently we have identified the primer for initiation of reverse transcriptase of M-MuLV RNA as $tRNA^{Pro}$ (Peters et al., 1977). The sequence of $tRNA^{Pro}$ is considerably different from that of the avian $tRNA^{Trp}$ (Harada et al., 1975, and in preparation). However, it does have in common with $tRNA^{Trp}$ the unusual sequence G-Ψ-Ψ-C instead of the usual G-T-Ψ-C sequence. This similarity prompted us to investigate whether AMV reverse transcriptase bound $tRNA^{Pro}$. [^{32}P]$tRNA^{Pro}$, purified from labeled NRK cells or from labeled M-MuLV virions by two-dimensional gel electrophoresis, is bound to AMV reverse transcriptase as determined by both the Sephadex G-100 assay and glycerol gradients (Table IV). In one experiment, the RNA that eluted from the Sephadex G-100 column in the void volume together with the enzymatic activity was identified as $tRNA^{Pro}$ by fingerprint analysis (unpublished data). We are unable to explain why this binding of $tRNA^{Pro}$ was not detected in earlier studies (Panet et al., 1975). AMV reverse transcriptase did not bind wwo other tRNA species purified from M-MuLV virions.

Heterologous Initiation. The AMV and M-MuLV enzymes carried out DNA synthesis using either avian or murine 70S RNA as template (Figure 3). With the same number of enzyme units, the kinetics of synthesis were similar using any combination of RNA and enzyme. This observation raised the question of what tRNA species served as the primer in heterologous reactions.

TABLE VII: Inability of Moloney MuLV Reverse Transcriptase to Bind $tRNA^{Pro}$ or $tRNA^{Trp}$.[a]

RNA	Source of RNA	Assay	% bound
$tRNA^{Pro}$	M-MuLV virions	Sephadex G-100	<2
$tRNA^{Pro}$	M-MuLV virions	Glycerol gradient	<2
$tRNA^{Pro}$	NRK cells	Sephadex G-100	<2
$tRNA^{Pro}$	NRK cells	Glycerol gradient	<2
$tRNA^{Trp}$	Chick cells	Sephadex G-100	<2
$tRNA^{Trp}$	Chick cells	Glycerol gradient	<2

[a] Reactions contained in 100 μL: 10 μg of Moloney MuLV reverse transcriptase and 1×10^4 cpm [^{32}P]tRNA (specific activity 2×10^6 cpm/μg) prepared as described in the Experimental Section.

To characterize the RNA primer used in the reactions in which RSV 70S RNA served as a template, reactions were carried out in the presence of [α-^{32}P]dATP alone. The RNA primers tagged with [^{32}P]dAMP were then purified and analyzed by two-dimensional polyacrylamide gel electrophoresis (Figure 4). ^{32}P-labeled 5S RNA was included as a marker on each gel. Both the M-MuLV and AMV enzymes used as a primer a single tRNA species whose electrophoretic mobility was identical with that of $tRNA^{Trp}$ (downward arrows in Figure 4). Similar experiments demonstrate that both enzymes use the murine primer $tRNA^{Pro}$ when M-MuLV 70S RNA is used as template (data not shown).

The Murine Enzyme does Not Tightly Bind tRNA. The ability of purified MuLV reverse transcriptase to bind $tRNA^{Pro}$ was tested by both the Sephadex G-100 and glycerol gradient assays. Table VII shows that the purified enzyme did not detectably bind to either $tRNA^{Pro}$ or $tRNA^{Trp}$, using the conditions suitable for the binding of $tRNA^{Trp}$ to AMV polymerase.

Duesberg et al. (1971) observed that RSV reverse transcriptase, as isolated directly from virions by disruption with a non-ionic detergent, sediments faster than the extensively purified reverse transcriptase. We have reported previously and have also shown here (Figure 2) that addition of a molar excess of either $tRNA^{Trp}$ or unfractionated chick cell tRNA increases the sedimentation of purified AMV reverse transcriptase (Panet et al., 1975). In contrast, the sedimentation velocity of the M-MuLV reverse transcriptase isolated by

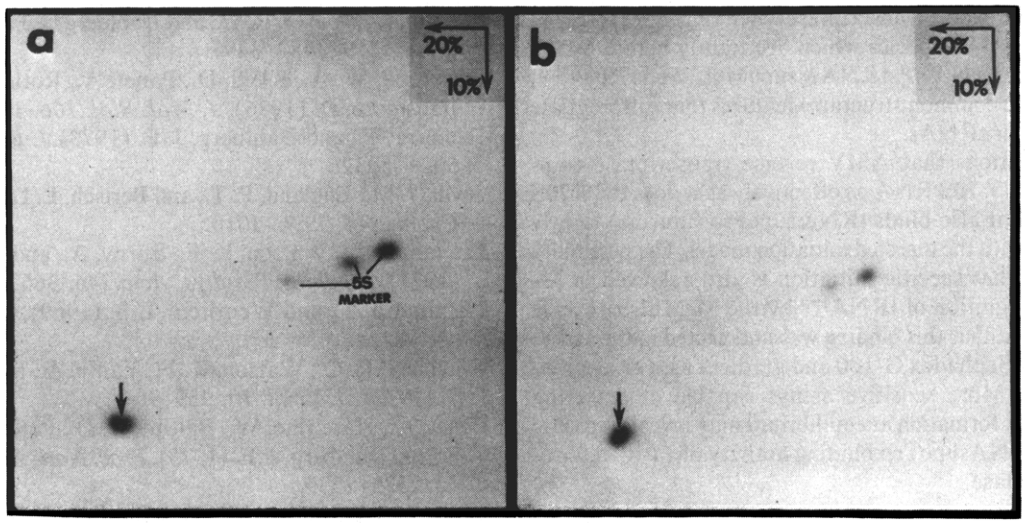

FIGURE 4: Initiation of reverse transcription of RSV 70S RNA by either avian or murine reverse transcriptase. Reverse transcription was performed using isolated 70S RNA from RSV as template·primer and purified AMV or M-MuLV DNA polymerases. [α-^{32}P]dATP was the only exogenously added triphosphate precursor. The reaction products were purified by phenol extraction and ethanol precipitation and analyzed by two-dimensional gel electrophoresis (Ikemura and Dahlberg, 1973). The products were heated to 95 °C for 2 min and rapidly cooled before application to the gel. A sample of 5S rRNA was included as marker. (a) RSV 70S RNA with M-MuLV DNA polymerase; (b) RSV 70S RNA with AMV DNA polymerase.

disruption of the virions with Nonidet P-40 was the same as that of the purified enzyme (Figure 5). Addition of a large molar excess of unfractionated murine cell tRNA did not increase the sedimentation velocity of this enzyme suggesting that the enzyme did not form a tight complex with $tRNA^{Pro}$.

Discussion

We have studied some parameters of the binding of $tRNA^{Trp}$ by AMV reverse transcriptase. The binding is not strongly influenced by the ionic strength of the binding and elution buffers. Divalent cations are not required; binding appears to be slightly better when magnesium ions are replaced by 1 mM EDTA.

Our studies on the binding of modified $tRNA^{Trp}$ by reverse transcriptase suggest that much of the tRNA structure is required for the binding reaction. The isolated fragments of $tRNA^{Trp}$ produced by cleavage of the molecule at the anticodon loop by limited S1 digestion or at the m^7G residue were not bound by the reverse transcriptase. However, $tRNA^{Trp}$ which is modified or elongated by deoxyribonucleotides at the 3′-OH end is bound by the enzyme.

It has not been possible to localize the $tRNA^{Trp}$ binding site in the $\alpha\beta$ dimer. The α subunit, which probably exists as a dimer in our reaction [it sediments faster than *E. coli* polymerase I which has a molecular weight of 110 000 (Jovin et al., 1969; Rogg et al., 1969)] does not bind $tRNA^{Trp}$ tightly nor does it initiate synthesis on 70S viral RNA efficiently. However, the α subunit does retain its ability to transcribe poly(RNA)·oligo(DNA) template·primer complexes. It is possible that the binding site for $tRNA^{Trp}$ is localized on the β subunit. If this were true, then the reduced activity of the α subunit on the 70S RNA template could be attributed to the failure of the α subunit to bind tightly to $tRNA^{Trp}$ that is annealed to the template. On the other hand, the reduced activity of the α subunit on 70S RNA may be due to the low affinity (relative to the holoenzyme) this subunit displays for template RNA molecules (Grandgenett and Green, 1974; Panet et al., 1974).

Binding of $tRNA^{Trp}$ by reverse transcriptase may serve to direct the enzyme to the correct initiation site on the 35S genome (the directed initiation model). $tRNA^{Trp}$ is hybridized to a unique site on the 35S RNA. Eiden et al. (1976), Cordell et al. (1976), and Harada, Peters, and Dahlberg (unpublished) find that only about 16 bases at the 3′-OH end of the $tRNA^{Trp}$ are hybridized to the viral RNA. Eiden et al. (1976) point out that the anticodon loop, the dihydrouridine loop, and the double-stranded region of the acceptor stem can assume a normal configuration (in the "L" shaped structure) in such a tRNA–35S RNA complex. These may be the regions recog-

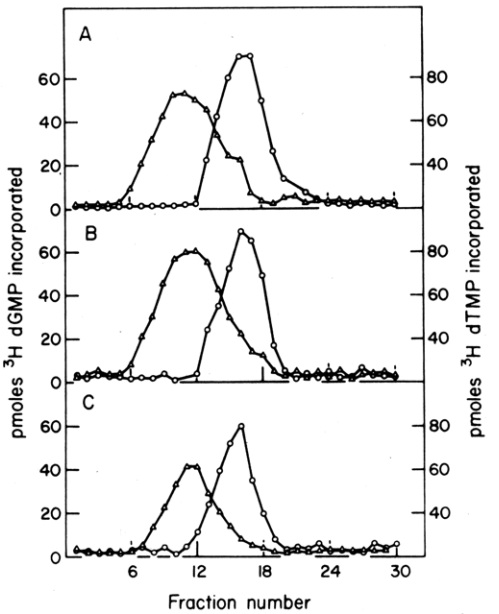

FIGURE 5: Moloney MuLV reverse transcriptase does not bind $tRNA^{Pro}$. Reactions in panels A and B contained 1 μg of M-MuLV purified reverse transcriptase and 6 units of *E. coli* DNA polymerase I in 100 μL. The reaction of panel B contained 20 μg of mouse liver tRNA (unfractionated). For panel C, 150 μg of purified M-MuLV virions in 100 μL was incubated for 15 min at 37 °C in the presence of 0.025% Nonidet P-40, 5 mM dithiothreitol, 5 mM $MgCl_2$, 25 mM NaCl, and 50 mM Tris-HCl (pH 7.5). The mixtures were layered onto 5-mL glycerol gradients. Centrifugation was for 34 h at 46 000 rpm in a Beckman SW 50.1 rotor and assayed for reverse transcriptase. (Δ—Δ) *E. coli* polymerase I; (O—O) reverse transcriptase.

nized by AMV reverse transcriptase when $tRNA^{Trp}$ is free in solution. The tRNA species which are tightly bound by the AMV enzyme ($tRNA^{Trp}$, tRNA&supcMet$_4$, and $tRNA^{Pro}$) must have some common structural features that differentiate them from other tRNAs.

The observations that AMV reverse transcriptase transcribes M-MuLV 70S RNA as effectively as it does RSV 70S RNA and that it also binds tRN&supcPro (murine) tightly are consistent with the directed initiation model. The possibility remains open that specific initiation is also achieved in M-MuLV by recognition of $tRNA^{Pro}$ by the M-MuLV reverse transcriptase but that this binding was undetected in the assays used. Both the Sephadex G-100 and gradient assays separate the reactants. More sensitive assays capable of detecting weaker complex formation at equilibrium may reveal a specific $tRNA^{Pro}$ or tRNAsupcTrp binding activity of M-MuLV reverse transcriptase.

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