JOURNAL OF VIROLOGY, May 1978, p. 214-220
0022-538X/78/0026-0214$02.00/0

Vol. 26, No. 2
Printed in U.S.A.

Binding of tRNA to Reverse Transcriptase of RNA Tumor Viruses

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Received for publication 28 November 1977

The interaction of tRNA with the reverse transcriptase (RNA-dependent DNA polymerase) of mammalian RNA viruses, such as Moloney murine leukemia virus and simian sarcoma virus, has been studied. Whereas the purified reverse transcriptase of mammalian viruses sedimented in glycerol gradients as a globular protein with a molecular weight of 70,000, after interaction with tRNA the enzyme cosedimented with a protein of 150,000 molecular weight. The twofold increase in molecular weight could be a result of either two reverse transcriptase molecules complexed with a tRNA or, alternatively, several tRNA molecules bound to a single enzyme polypeptide. The enzyme complexes were dissociated in part upon degradation of the tRNA moiety by pancreatic RNase A. The reverse transcriptase released from virions of Moloney murine leukemia virus, simian sarcoma virus, and avian myeloblastosis virus, by nonionic detergent, migrated faster on glycerol gradients than purified enzyme preparation. This phenomenon was probably due to complex formation between part of the virion enzyme and the tRNA, which is endogenous in virions. Addition of exogenous tRNA was needed, however, to quantitatively complex all the virion reverse transcriptase of Moloney murine leukemia virus and simian sarcoma viruses. The reverse transcriptase of Moloney murine leukemia virus did not show tRNA species specificity in the binding reaction when glycerol gradients were used for assay. Thus, several tRNA species of *Escherichia coli*, yeast, chicken, and rat origin were able to complex with the enzyme. The species specificity in the interaction between tRNA and avian myeloblastosis virus reverse transcriptase was also examined. We demonstrated that under our experimental conditions, this enzyme binds different tRNA species of *E. coli* and yeast as well as tRNA of chicken origin.

Initiation of DNA synthesis in RNA tumor viruses is primed in vitro by a tRNA, which is part of the 70S RNA genome complex (1). Two groups of virus use different tRNA species of cell origin as primer. Thus, tryptophan tRNA is the primer in avian myeloblastosis virus (AMV) and avian sarcoma virus (6), and proline tRNA has recently been shown to be the primer in Moloney murine leukemia virus (MuLV) (17). The primer $tRNA^{Trp}$ in AMV has two recognition sites: it is bound at a specific site to the template 35S RNA genome subunit (21), and it complexes with high affinity to the reverse transcriptase (16). The binding of $tRNA^{Trp}$ to purified AMV reverse transcriptase requires the holoenzyme, which consists of two polypeptides (α and β). The isolated smaller α subunit (molecular weight, 65,000), which manifests both DNA polymerase and RNase H activities (4), cannot form a stable complex with $tRNA^{Trp}$ (5, 7). Reverse transcriptase isolated from mammalian RNA tumor viruses is a single polypeptide with a molecular weight of 70,000 (19). This polypeptide, like the α subunit of AMV reverse transcriptase, shows both DNA polymerase and RNase H activities (12, 22).

In this study we show that the reverse transcriptase of two mammalian viruses, Moloney MuLV and simian sarcoma (SiSV), forms complexes with tRNA. The binding of tRNA changes the molecular weight of the mammalian enzyme from 70,000 to 150,000, suggesting a complex structure of two enzyme subunits and a tRNA molecule.

MATERIALS AND METHODS

Viruses and enzymes. Moloney MuLV was grown in Swiss mouse cells (clone 1) and purified as described before (3). AMV was a gift from J. W. Beard of Life Sciences Inc., St. Petersburg, Fla., and SiSV was obtained from J. Gruber of the National Institutes of Health, Bethesda, Md. Reverse transcriptase was purified from these three viruses by sequential column chromatography on DEAE-Sephadex followed by phosphocellulose (23). All buffers used throughout the purification procedure contained 20 mM potassium phosphate, pH 7.5, rather than Tris-hydrochloride, pH 7.9, since we found that phosphate buffers significantly increased the stability of the reverse transcriptase

during purification. The purified enzyme was dialyzed extensively against 50 mM Tris-hydrochloride (pH 7.5), 0.1 M KCl, 0.1% (vol/vol) Triton X-100, 20% (vol/vol) ethylene glycol, 2% (vol/vol) glycerol, and 10 mM mercaptoethanol. Pancreatic RNase A, electrophoretically purified, was purchased from Worthington Biochemicals Co., Freehold, N.J.

Reagents. Chicken liver tRNA and rat liver tRNA were isolated as described by Rogg et al. (18). Yeast tRNA was a product of Schwarz/Mann, Orangeburg, N.Y., and *Escherichia coli* W tRNA was a gift from Y. Lapidot, The Hebrew University. These tRNA preparations were further purified on DEAE-cellulose columns (Whatman, DE-52). The tRNA was loaded onto the columns, which were then washed stepwise with potassium acetate buffer, pH 5.0, containing 0.1 and 0.3 M NaCl. The tRNA was then eluted with 1.0 M NaCl and precipitated with 2 volumes of ethanol.

Purified *E. coli* $tRNA_1^{Val}$ and yeast $tRNA^{Phe}$ were products of Boehringer Chemical Co., Mannheim, Germany. These purified tRNA preparations were found to lack acceptor tRNA for proline, tryptophan, and methionine. One absorbance unit of tRNA at 260 nm was assumed to be 50 μg, or 1.67 nmol. Poly(A) and $poly(dT)_{10}$ were gifts from Y. Lapidot; [^{3}H]dTTP, 48 Ci/mmol, was obtained from the Radiochemical Centre, Amersham, England; bovine serum albumin, electrophoretically purified, was from Calbiochem, Los Angeles, Calif.; and purified human immunoglobulin G (IgG) was a gift from G. Spira, The Hebrew University.

DNA polymerase assay. Reaction mixtures (100 μl) contained 50 mM Tris-hydrochloride (pH 8.3), 10 mM dithiothreitol, 1 μg of poly(A) · oligo(dT), 10 μg of bovine serum albumin, 6 mM $MgCl_2$, 10 μM [^{3}H]dTTP (350 cpm/pmol), and samples (usually 30 μl) from the glycerol gradient. Reaction mixtures were incubated for 60 min at 37°C, and trichloroacetic acid-insoluble radioactivity was collected on glass fiber filters (Whatman). One unit of activity is the amount of reverse transcriptase needed to catalyze the incorporation of 1 pmol of [^{3}H]dTMP per min under the above conditions.

tRNA binding assay. Mixtures (200 μl) contained 50 mM Tris-hydrochloride (pH 7.5), 5 mM $MgCl_2$, 10 mM dithiothreitol, and 0.2% (vol/vol) Triton X-100. After 10 min of incubation at 4°C, the internal protein markers bovine serum albumin (0.6 mg) and human IgG (0.6 mg) were added, and the mixtures were layered onto 5-ml glycerol gradients (5 to 25%, vol/vol) in 50 mM Tris-hydrochloride (pH 7.5), 0.1 M KCl, 0.1% (vol/vol) Triton X-100, 10 mM mercaptoethanol, and 20% (vol/vol) ethylene glycol. Gradients were centrifuged for 27 h at 49,000 rpm at 4°C in an SW 50.1 rotor of a Beckman ultracentrifuge and fractionated from the bottom (usually 32 0.16-ml fractions were collected). Samples (30 μl) were taken for the DNA polymerase assay, and 30 μl was taken for protein determination (11). Proteins were first precipitated with 10% (vol/vol) trichloroacetic acid to remove materials that interfere with the Lowry assay (11).

RESULTS

Binding of tRNA to Moloney MuLV reverse transcriptase. To study the interaction between enzyme and tRNA, we first sought conditions to separate the complex from the unbound reverse transcriptase. Glycerol gradients containing ethylene glycol were found to be most suitable for this purpose. Rat liver tRNA was mixed with purified enzyme, and after short incubation in the cold the mixture, together with two internal protein markers, was layered onto the gradient. After centrifugation for 27 h, the positions in the gradient of the reverse transcriptase and the protein markers bovine serum albumin and human IgG were determined. Figure 1 shows that tRNA affects the sedimentation coefficient of the reverse transcriptase. Whereas free enzyme cosedimented with bovine serum albumin (4.7S; molecular weight, 70,000), addition of 30 μg of tRNA shifted the polymerase activity to the position of the IgG marker (7.2S; molecular weight, 156,000). A twofold increase in sedimentation rate of the enzyme cannot be accounted for by equimolar binding of one enzyme molecule (molecular weight, 70,000) with a single tRNA molecule (molecular weight, 25,000). Such a complex is more likely to consist of two enzyme polypeptides and one tRNA molecule or, alternatively, several tRNA molecules bound to a single polypeptide. Addition of an even larger excess of tRNA does not further increase the sedimentation rate of the enzyme (data not shown). However, smaller amounts of tRNA (3 μg) cause the majority of reverse transcriptase molecules to migrate at an intermediate position between the two internal protein

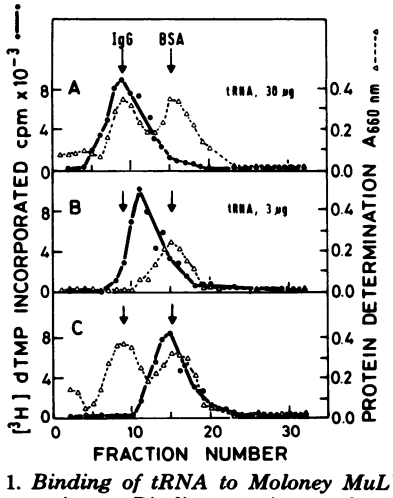

FIG. 1. *Binding of tRNA to Moloney MuLV reverse transcriptase. Binding reactions and centrifugation in a glycerol gradient were performed as described in the text. (A) Forty units of reverse transcriptase and 30 μg of rat liver tRNA; (B) 40 units of reverse transcriptase and 3 μg of rat liver tRNA; (C) 40 units of reverse transcriptase. $A_{660\,nm}$, Absorbance at 660 nm.*

markers (Fig. 1B). The formation of an apparently smaller enzyme complex may either be the result of poor separation between the complex and free enzyme or perhaps limited amounts of tRNA induce formation of a smaller complex. Inclusion of only bovine serum albumin as a marker in the gradient (Fig. 1B) demonstrates that the purified reverse transcriptase does not contribute enough protein to interfere with the exact positioning of the markers.

To prove that the reverse transcriptase complex indeed contains tRNA, we treated such complexes with pancreatic RNase A, followed by centrifugation in glycerol gradients. RNase A was mixed with tRNA either before (Fig. 2C) or after (Fig. 2B) binding to the enzyme, and in both experiments most of the reverse transcriptase sedimented as free enzyme. Some of the complexes after RNase treatment sedimented faster than the free reverse transcriptase (the position of bovine serum albumin marker) and similar to complexes made with a small amount of tRNA (Fig. 1B). These enzyme molecules may still contain some intact, or partially degraded, tRNA molecules, since under our experimental conditions 5 to 10% of the tRNA was not converted into acid-soluble oligonucleotides by RNase A. The fact that RNase A dissociates preformed complexes (Fig. 2B) suggests that the reverse transcriptase-bound tRNA is not protected from RNase A action.

Specificity of the binding reaction. We have studied tRNA species specificity in binding to the reverse transcriptase of Moloney MuLV and AMV by using unfractionated or purified tRNA species of different organisms. Figure 3 shows that Moloney MuLV reverse transcriptase can interact with unfractionated chicken liver tRNA, yeast tRNA, or *E. coli* tRNA with a subsequent increase in the sedimentation rate of the enzyme. Each of these tRNA preparations contains proline acceptor species, but the nucleotide sequence of $tRNA^{Pro}$ of mammalian origin, which serves as a primer for DNA synthesis in Moloney MuLV (17), differs from that of *E. coli* $tRNA^{Pro}$ (J. Dahlberg, personal communication). Pure preparations of yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Val}$, which contain no proline and tryptophan acceptor tRNA, also change the sedimentation coefficient of the reverse transcriptase (Fig. 4).

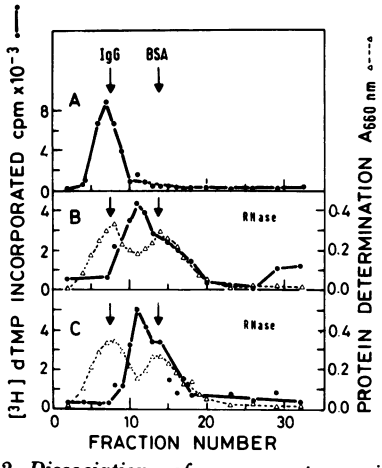

FIG. 2. *Dissociation of reverse transcriptase-tRNA complexes by RNase A. Binding reactions with Moloney MuLV reverse transcriptase (40 units) and glycerol gradient centrifugation were carried out as described in the text. (A) Enzyme and 50 μg of rat liver tRNA. (B) Enzyme and 50 μg of rat liver tRNA were incubated for 10 min at 4°C, and then RNase A (10 μg) was added. After 30 min at 4°C, dithiothreitol was added to 10 mM, and the mixture was loaded onto a gradient. (C) A 50-μg amount of rat liver tRNA was preincubated for 15 min at 37°C with RNase A (10 μg) in the binding mixture, and then reverse transcriptase was added. After an additional 10 min at 4°C, the mixture was layered onto a gradient. $A_{660\,nm}$, Absorbance at 660 nm.*

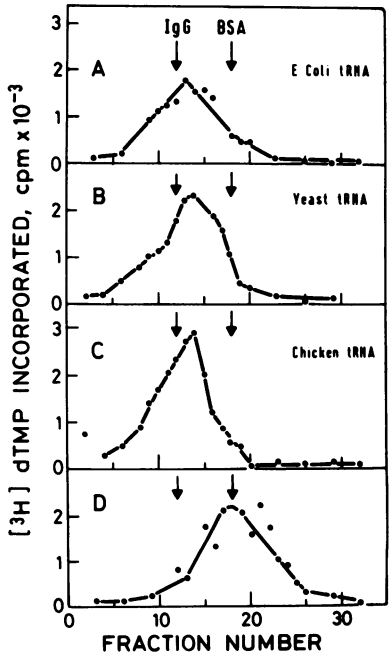

FIG. 3. *Reverse transcriptase complex formation with unfractionated tRNA of various organisms. Conditions for binding reactions with Moloney MuLV reverse transcriptase (40 units) and glycerol gradient centrifugation are described in the text. (A) Enzyme and 29 μg of E. coli tRNA; (B) enzyme and 34 μg of yeast tRNA; (C) enzyme and 33 μg of chicken liver tRNA; (D) enzyme.*

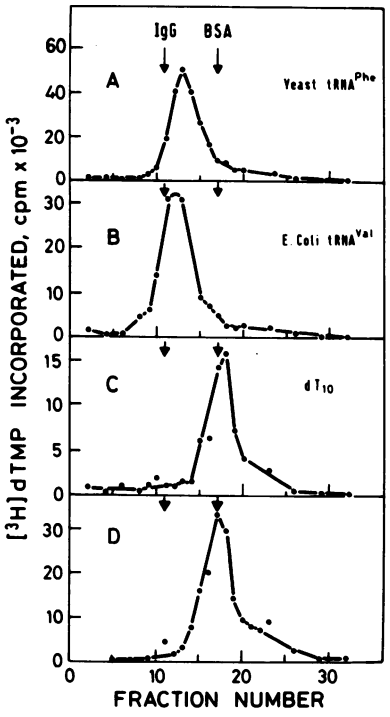

FIG. 4. *Binding of reverse transcriptase to purified tRNA species. Conditions for binding reactions with Moloney MuLV reverse transcriptase (60 units) and glycerol gradient centrifugation are described in the text. (A) Enzyme and 20 μg of yeast tRNA^{Phe}; (B) enzyme and 25 μg of E. coli tRNA^{Val}; (C) enzyme and 10 μg of oligo(dT); (D) enzyme.*

From these results we conclude that under the binding conditions used, Moloney MuLV reverse transcriptase does not selectively bind a unique tRNA species. In contrast, synthetic oligo(dT), which is an efficient primer for DNA synthesis with poly(A) as template, does not affect the sedimentation rate of the enzyme (Fig. 4).

To further investigate the question of specificity, we reexamined the tRNA binding specificity of AMV reverse transcriptase by the use of glycerol gradients. In a previous study (16) it was shown that out of unfractionated chicken cell [^{32}P]tRNA, only two species, tRNA^{Trp} and tRNA$_4^{Met}$, were able to bind to AMV enzyme with high affinity. The analysis of complex formation was carried out by filtration of the mixtures through Sephadex G-100 columns that separated the enzyme-bound [^{32}P]tRNA from unbound [^{32}P]tRNA (molecular weight, 25,000). When glycerol gradient centrifugation, rather than gel filtration, was used for analysis of AMV reverse transcriptase-tRNA complex formation (Fig. 5), we found that the AMV polymerase could also bind tRNA species other than tRNA^{Trp} and tRNA$_4^{Met}$ of mammalian origin. Thus, total yeast tRNA, which contains tRNA^{Trp} with different primary structures (6, 8, 10) or pure *E. coli* tRNA^{Val} and yeast tRNA^{Phe}, can complex with AMV reverse transcriptase and change its sedimentation coefficient from 7.2S (IgG marker) to 9S (Fig. 5).

Structure of reverse transcriptase in disrupted virions of Moloney MuLV, SiSV, and AMV. Some 30% of the total RNA in virions of Moloney MuLV consists of small RNA (4–7S) containing 15 species of host cell tRNA (20). Some of the small RNA molecules are bound in the 70S RNA complex, and the rest are released free in solution after disruption of virions with nonionic detergent. We have investigated whether the "free tRNA" is bound to the reverse transcriptase, which is also released from virions by detergent treatment. The results in Fig. 6 illustrate that the enzyme in disrupted Moloney MuLV migrates in glycerol gradients faster than bovine serum albumin, which marks the position of the purified reverse transcriptase. Addition of exogenous rat liver tRNA further increases the molecular weight of virion polymerase, with the

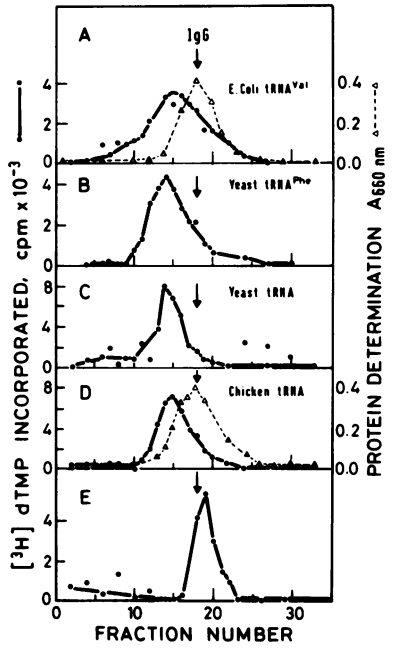

FIG. 5. *Interaction of various tRNA species with AMV reverse transcriptase. Conditions for binding reaction and glycerol gradient centrifugation with AMV reverse transcriptase (50 units) are described in the text. (A) Enzyme and 20 μg of E. coli tRNA^{Val}; (B) enzyme and 15 μg of yeast tRNA^{Phe}; (C) enzyme and 52 μg of total yeast tRNA; (D) enzyme and 47 μg of total chicken liver tRNA; (E) enzyme. A$_{660\ nm}$, Absorbance at 660 nm.*

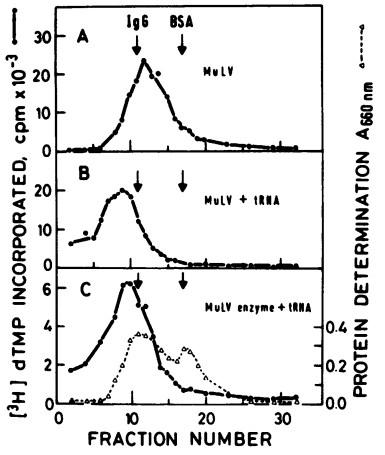

FIG. 6. *Binding of tRNA to reverse transcriptase in virions. Conditions for binding reactions are described in the text. (A) Moloney MuLV (250 μg) was treated with 0.2% (vol/vol) Triton X-100 in a binding mixture. After incubation for 10 min at 4°C, the mixture was centrifuged for 20 min at 6,000 × g, and the clear supernatant was layered onto a glycerol gradient. (B) Virions (250 μg) were treated with Triton X-100 as described in (A) in the presence of 300 μg of rat liver tRNA. (C) Forty units of purified Moloney MuLV reverse transcriptase and 30 μg of rat liver tRNA. Protein determinations were not done with gradients A and B, since virion proteins interfere with the assay of the markers. $A_{660\ nm}$, Absorbance at 660 nm.*

result that it sediments at the position of the purified reverse transcriptase-tRNA complex (Fig. 6B and C). These observations suggest that some polymerase molecules released from virions are complexed with tRNA and thus migrate faster in glycerol gradients. However, to quantitatively complex all of the virion enzyme, additional exogenous tRNA is needed. It is presently unknown whether the enzyme is bound to tRNA in the virion or complexes are formed subsequent to virus disruption.

To determine whether mammalian RNA tumor virus reverse transcriptase other than the Moloney MuLV enzyme can also bind tRNA, we chose a primate virus, SiSV. The results presented in Fig. 7 show that whereas purified SiSV reverse transcriptase migrates as a protein with a molecular weight of 70,000 (using bovine serum albumin as marker), the enzyme released from SiSV virions is somewhat heavier (Fig. 7A). After addition of exogenous tRNA, the DNA polymerase of SiSV sediments as a protein of 150,000 molecular weight (Fig. 7B). This experiment demonstrates that the reverse transcriptase of a primate virus can bind tRNA similarly to the enzyme of a murine virus.

We have further investigated the structure of the reverse transcriptase in virions of AMV. Figure 8A shows that all the reverse transcriptase released from AMV after detergent treatment migrates on glycerol gradients as a protein with a sedimentation coefficient of 9S, consistent with a complex of enzyme (7.2S) and tRNA (Fig. 8C and D). Addition of exogenous tRNA to the virus does not further increase the sedimentation rate of the complex (Fig. 8B). It is interesting to note that a virion of AMV contains between 20 and 80 molecules of reverse transcriptase (14, 15) and some 35 to 70 molecules of 15 tRNA species; of these, only 7 to 10 molecules are the primer $tRNA^{Trp}$ (20). The observation that all the released virion enzyme is complexed to tRNA further strengthens the notion that many tRNA species, besides $tRNA^{Trp}$, can bind to the reverse transcriptase when glycerol gradients are being used as an assay system.

DISCUSSION

In this study we have shown that the reverse transcriptase of mammalian RNA tumor viruses exists in two forms; the purified enzyme is a single polypeptide with a molecular weight of 70,000, which is converted into a high-molecular-weight complex after binding of tRNA. The exact structure of this complex is not yet known, but according to its molecular weight (150,000) two arrangements are possible: (i) an aggregate

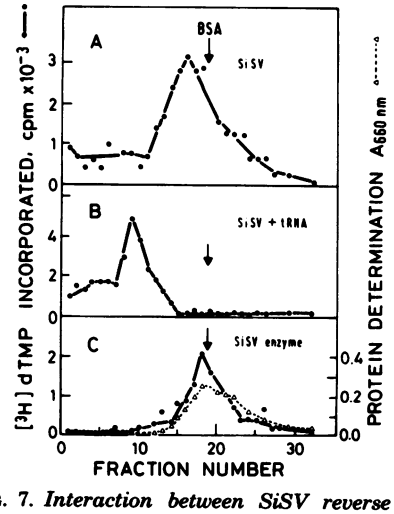

FIG. 7. *Interaction between SiSV reverse transcriptase and tRNA. Conditions for binding reactions and glycerol gradient centrifugation are described in the text. (A) SiSV (125 μg) was treated with Triton X-100 and layered onto a glycerol gradient as described in the legend to Fig. 6; (B) SiSV (125 μg) was treated with Triton X-100 in the presence of 300 μg of rat liver tRNA as described in the legend to Fig. 6; (C) purified SiSV reverse transcriptase (25 units). $A_{660\ nm}$, Absorbance at 660 nm.*

of two subunits with one or two tRNA molecules, or (ii) several tRNA molecules bound to a single polypeptide. If the mammalian virus reverse transcriptase-tRNA complex is indeed a dimer, then it may be compared to the holoenzyme of avian sarcoma leukosis viruses. The avian holoenzyme is composed of two subunits, α and β (4), or β,β in Rous sarcoma virus propagated in duck cells (9). In experiments in which tRNA concentrations were low, the reverse transcriptase complex sedimented at an intermediate position. Such sedimentation may reflect formation of a complex with a different structure or dissociation of the dimer during centrifugation.

It may be argued that the association of reverse transcriptase and tRNA represents nonspecific aggregation of protein and nucleic acid; there are, however, several lines of evidence that indicate specificity in this interaction. (i) Complex formation requires intact tRNA, whereas fragments of tRNA (7), oligo(dT), or 16S rRNA of *E. coli* (unpublished observation) do not bind to the enzyme. (ii) The pattern of the sedimenting enzyme complex suggests a unique species with a molecular weight of 150,000; nonspecific aggregation is more likely to produce multiple species. (iii) Proteins such as bovine serum albumin, IgG, or even the isolated α subunit of AMV reverse transcriptase (5, 7) do not complex with tRNA. (iv) Reverse transcriptase of Rous sarcoma virus temperature-sensitive mutant *ts*335 is thermolabile for both DNA polymerase activity (24) and the tRNA binding function (A. Panet, G. Weil, and R. Friis, manuscript in preparation). (v) Inhibitors of reverse transcriptase activity such as monospecific antibody and *N*-ethylmaleimide also inhibit the tRNA binding function (7).

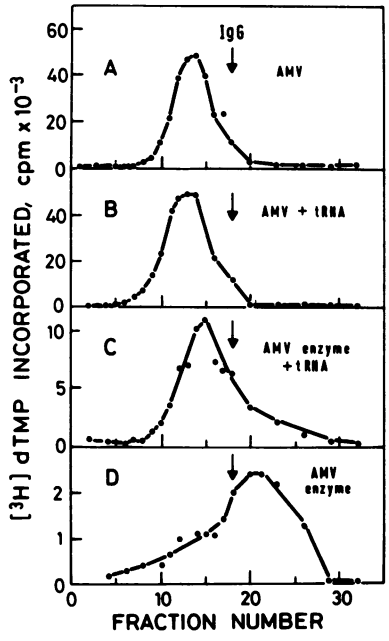

FIG. 8. *Binding of tRNA to reverse transcriptase in disrupted virions of AMV. Conditions for tRNA binding are described in the text. IgG marker was added to reactions (C) and (D) only, and glycerol gradients were centrifuged for 20 h. (A) AMV (500 μg) was treated with Triton X-100 as described in the legend to Fig. 6; (B) AMV (500 μg) was treated with Triton X-100 in the presence of 125 μg of chicken liver tRNA as described in the legend to Fig. 6; (C) purified AMV reverse transcriptase (50 units) and 50 μg of chicken liver tRNA; (D) purified AMV reverse transcriptase (50 units).*

The fact that crude reverse transcriptase released from virions sediments faster than purified enzyme suggests that it is bound to tRNA, and, during enzyme purification, the tRNA is removed and the two-subunit enzyme of mammalian viruses dissociates. On the other hand, the two subunits of the avian virus enzyme may hold together even without tRNA. It is not clear to us why in a previous study mouse liver tRNA did not interact with MuLV reverse transcriptase (7). The quality of the tRNA preparation or the different composition of the glycerol gradients used for the assay may have been responsible for the seeming lack of interaction.

There are several studies in the literature regarding the structure of the reverse transcriptase in virions and cells. Mondal et al. (13) have reported the existence of two enzymes in lymphosarcoma cells productively infected with gibbon ape leukemia virus: (i) an enzyme similar to the reverse transcriptase isolated from virions, with a molecular weight of 70,000; and (ii) a high-molecular-weight enzyme (140,000) that is able to copy the 70S RNA genome very efficiently. This latter form could be dissociated by high-salt and detergent treatment to yield the 70,000-molecular-weight enzyme. Based on the results presented here, we suggest that the high-molecular-weight reverse transcriptase found in productively infected cells (13) or in Rous sarcoma virus-disrupted virions (2) is indeed an enzyme complexed to tRNA. The association of tRNA with the reverse transcriptase in productively infected cells could provide the mechanism responsible for the incorporation of certain cell tRNA species into the budding virus. Such a mechanism, however, required some tRNA species specificity in the binding reaction, since a specific subset of the cell tRNA's is incorporated into virions (20).

AMV reverse transcriptase was shown to bind only $tRNA^{Trp}$ and $tRNA_4^{Met}$ with high affinity (16). In the present study we found that the

AMV enzyme can also bind other tRNA species, although possibly with lower affinity. Such binding was observed when high concentrations of tRNA were added to the purified AMV enzyme and complex formation was assayed using glycerol gradient centrifugation rather than Sephadex gel filtration (16). This apparent lack of specificity is reminiscent of the interaction between aminoacyl-tRNA synthetases and tRNA. The latter enzymes bind their cognate tRNA's tightly but can also associate with other tRNA species with lower affinity (25). Furthermore, we could not demonstrate tRNA species specificity in the complex formed with MuLV reverse transcriptase. If indeed the affinity of the MuLV enzyme to its primer $tRNA^{Pro}$ is higher than to other tRNA species, other experimental conditions will have to be sought. Sephadex gel filtration, for example, failed to show any complex formation between [^{32}P]tRNA and the MuLV reverse transcriptase.

ACKNOWLEDGMENTS

This work was supported by grants from Stiftung Volkswagenwerk and The Israel Commission for Basic Research.

We thank N. Goldblum for his help.

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