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Binding of Tryptophanyl-tRNA to the Reverse Transcriptase of Replication-Defective Avian Sarcoma Viruses

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The ability of reverse transcriptase to bind to [^{3}H]tryptophanyl-tRNA and to function as DNA polymerase was compared for five temperature-sensitive mutants of avian sarcoma virus. Both activities of the reverse transcriptase were found to be heat labile in LA 335 and LA 336 as compared with the wild-type parents. For the other mutant viruses, LA 338, LA 343, and LA 672, grown at the permissive temperature, the reverse transcriptase was nearly as heat stable as for the wild-type parents in terms of tRNA binding and DNA polymerase. LA 338, LA 343, and LA 672 showed characteristic defects in their reverse transcriptase when propagated at the nonpermissive temperature; namely, tryptophanyl-tRNA binding and DNA polymerase activities were coordinately decreased in these virions. The reduced enzymatic activities were not entirely due to an inactive reverse transcriptase present in the virions, however, but rather lower amounts of enzyme protein incorporated into the virions contributed to the effect, according to assays of reverse transcriptase antigen by radioimmune competition.

Three activities can be independently measured with purified reverse transcriptase of avian sarcoma viruses (ASV), namely, DNA polymerase (1, 20), RNase H (13), and tryptophan-tRNA binding (16). Binding of the primer tryptophan-tRNA is viewed as an initiation function in proviral-DNA synthesis, whereas DNA polymerase activity with the polyadenylate·oligodeoxythymidylate [poly(A)·oligo(dT)] template is considered an elongation function (19, 23).

Several replication-defective ASV have been investigated for their reverse transcriptase activities (12, 24, 25). Early replication temperature-sensitive (*ts*) mutants LA 335 and LA 337 were shown to contain heat-labile reverse transcriptase for both DNA polymerase and RNase H, thus indicating that these enzymatic activities are coded by the viral genome. RSV_{α}, a nonconditional defective mutant, has been shown to completely lack the reverse transcriptase protein (8, 15).

In the present study, we have examined the DNA-polymerase activity and the binding to tryptophanyl (trp)-tRNA of reverse transcriptase from five conditional ASV mutants, two mutants carrying an early replication lesion and three mutants defective in a late replication function.

MATERIALS AND METHODS

Materials. [^{3}H]tryptophan, 11 Ci/mmol, and [^{3}H]-dTTP, 46 Ci/mmol, were obtained from the Radiochemical Centre, Amersham, England; Sephadex G-100 was a Pharmacia Fine Chemicals product. Rat serum specific for purified avian myeloblastosis virus (AMV) reverse transcriptase was a kind gift from R. Nowinski. The immunoglobulin G (IgG) fraction was purified as described by Watson et al. (26). Chicken liver tRNA was prepared according to Rogg et al. (18). Chicken liver aminoacyl-tRNA synthetases were purified by using the procedure of Nishimura and Weinstein (14). AMV reverse transcriptase was purified from sucrose gradient-banded virus as previously described (17).

Preparation of [^{3}H]trp-tRNA. The reaction mixture (1 ml) contained: 20 mM Tris-hydrochloride, pH 7.5; 10 mM $MgCl_2$; 10 mM KCl; 2 mM ATP; 0.5 mM CTP; 2 mM dithiothreitol; 160 μCi of [^{3}H]tryptophan; 1 mg of chicken liver tRNA; and aminoacyl-tRNA synthetase preparation (260 μg). After 30 min of incubation at 37°C, sodium acetate buffer (pH 5.0) was added to a 0.1 M concentration; EDTA was added to 25 mM, and the tRNA was extracted three times with an equal volume of phenol saturated with 0.1 M sodium acetate (pH 5). The tRNA was precipitated by adding 2 volumes of ethanol, and the precipitate was washed twice by resuspending in 66% ethanol-33% sodium acetate (0.1 M), pH 5. The final pellet was dissolved in 10 mM sodium acetate (pH 5)-0.1 mM EDTA. A maximum of 0.4 to 0.5% of the crude chicken liver tRNA was routinely charged with [^{3}H]tryptophan.

DNA polymerase assay. Reaction mixtures (100 μl) contained: 50 mM Tris-hydrochloride, pH 8.3; 6 mM $MgCl_2$; 10 mM dithiothreitol; 10 μg of bovine serum albumin; 10 μM [^{3}H]dTTP (specific activity, 450 cpm/pmol); 0.5 μg of poly(A)·oligo(dT); and 20-

to 30-μl samples of reverse transcriptase. Incubations were for 30 min at 37°C. Reactions were stopped with 5% trichloroacetic acid, and radioactive precipitates were collected on glass fiber filters. A unit is the amount of enzyme needed to catalyze the incorporation of 1 pmol of [^{3}H]dTMP in 1 min under standard conditions.

Binding assay. Reaction mixtures (100 μl) contained: 50 mM Tris-hydrochloride, pH 7.5; 60 mM NaCl; 6 mM $MgCl_2$; 10 μg of bovine serum albumin; 0.2% Triton X-100; 5 mM dithiothreitol; 16,000 cpm of [^{3}H]trp-tRNA (unfractionated) (1.3 pmol of [^{3}H]tryptophan); and purified virus as indicated. After 10 min of incubation at 22°C, the mixtures were loaded onto Sephadex G-100 columns (0.6 by 22 cm) and eluted with buffer A (0.1 M potassium phosphate, pH 7.1; 10% glycerol; 0.1% Triton X-100; 10 mM mercaptoethanol). Fractions of 0.18 ml were collected; 20-μl samples were taken for assay of DNA polymerase activity, and the rest were counted for [^{3}H]trp-tRNA in toluene scintillation fluid containing 30% Triton X-100.

Cells and viruses. Chicken embryo cells were prepared from fertile white leghorn embryos obtained from Heisdorf and Nelson Farms, Redmond, Wash., and tested for the expression of chick helper factor according to the procedure of Friis et al. (5). Eagle Dulbecco-modified medium supplemented with 10% tryptose phosphate broth, 5% calf serum, and 1% dimethyl sulfoxide was used for virus stock preparation. Chicken helper factor-negative chicken embryo cells were infected with the various viruses in the presence of 2 μg of Polybrene (Aldrich Chemicals) per ml (21), and cultures were maintained with regular transfers for 10 to 14 days until 30 to 40 dishes (90 mm) of each wild type or mutant virus stock had been prepared. Harvests of virus were then made, usually at 24-h intervals, but, as reported below for *ts*-672, occasionally at 3-h intervals, from cultures maintained separately at 35 and 42°C. Cultures maintained at 42°C were harvested in a warm room on a warm table held at 42°C. Pooled culture supernatants were frozen at −70°C until a volume of 3 to 5 liters was available, at which time virus was pelleted in a Spinco type 19 rotor (19,000 rpm for 2 h). All the viruses tested in this work were purified under identical conditions by gradient purification on continuous 20 to 50% sucrose gradients. Biological controls were performed with all virus preparations to demonstrate that the stocks exhibited the typical temperature-sensitive properties of the mutants and the correct subgroup.

The following mutants obtained from the Prague strain (PR) of Rous sarcoma virus (RSV) have been used: *ts* LA 335, subgroup C (10); *ts* LA 338, subgroup C (27); *ts* LA 343, subgroup C (27); and *ts* LA 672, subgroup A (3). For control purposes, wild-type PR RSV, subgroup C (wt PR-C), has been used. TS LA 336 (formerly called *ts*149) (6, 22) was also investigated and its parent, wild-type ASV strain Bratislava 77, subgroup C (wt B77), was used for control purposes.

RESULTS

Binding of trp-tRNA to reverse transcriptase using disrupted virions. In previous studies, the interaction of the primer [^{32}P]-tRNA^{trp} and AMV reverse transcriptase has been demonstrated, using purified enzyme preparations (7, 16). To be able to measure tRNA complex formation with the enzyme of ASV temperature-sensitive mutants, we sought conditions for binding in crude virion extracts. Figure 1 demonstrates that [^{3}H]trp-tRNA binds to reverse transcriptase in detergent-disrupted virions as well as to a purified AMV reverse transcriptase preparation. Complex formation is evident by the comigration of virion DNA polymerase and part of the [^{3}H]trp-tRNA, with a sedimentation coefficient of 9S on glycerol gradients. Under the same conditions, unbound purified reverse transcriptase (not shown) has a sedimentation coefficient of 7.2S (150,000 molecular weight), similar to that of the protein marker IgG (16).

To exclude the possibility that [^{3}H]trp-tRNA binds in detergent-disrupted virions to proteins other than the polymerase and forms a complex with a sedimentation coefficient similar to that of the reverse transcriptase, we investigated whether known inhibitors of the viral DNA polymerase activity also inhibit tRNA complex formation in disrupted virions. In these experiments binding of [^{3}H]trp-tRNA was assayed by gel chromatography on Sephadex G-100, where the enzyme complex migrates in the void volume (fractions 13 to 15), while unbound [^{3}H]trp-tRNA elutes as the second peak (fractions 20 to 30) and free [^{3}H]tryptophan, a deacylation product from tRNA, is retarded to fractions 30 to 40 (Fig. 2). Preincubation of detergent-disrupted virions with monospecific antibody, produced in rats against purified reverse transcriptase (26), or with *N*-ethylmaleimide (NEM), a potent inhibitor of DNA polymerase and tRNA binding activities in purified enzyme preparations (15), abolished both activities in crude extracts (Fig. 2). These results suggest that of all viral proteins only the reverse transcriptase is able to form a specific and stable complex with trp-tRNA.

In this work [^{3}H]trp-tRNA rather than [^{32}P]-tRNA^{trp} was used to quantitate complex formation with reverse transcriptase, and we find several advantages in using the aminoacylated tRNA as a substrate: (i) specific tagging of crude tRNA preparation with [^{3}H]tryptophan is easy and does not require use of two-dimensional gel electrophoresis, which is necessary for the purification of [^{32}P]tRNA^{trp} from a mixture of tRNA's; (ii) the isotope in [^{3}H]trp-tRNA is stable as compared with the in vivo labeled [^{32}P]-tRNA, and specific activities of [^{3}H]trp-tRNA preparations are accurately assessed. The one disadvantage in using [^{3}H]trp-tRNA is the relative instability of the aminoacyl ester bond. We therefore ran glycerol gradient centrifugations

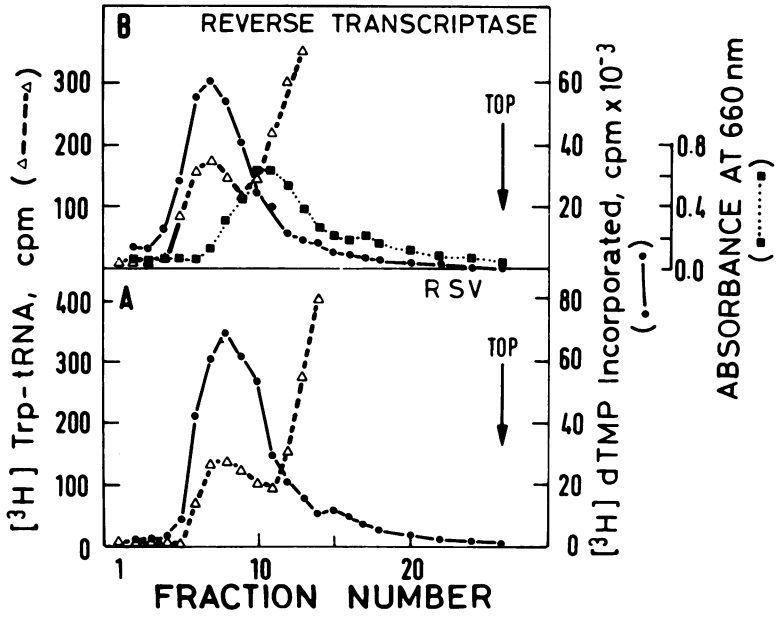

FIG. 1. *Binding of trp-tRNA in disrupted virions. Binding of [^{3}H]trp-tRNA was conducted as described in the text, except 50 mM potassium phosphate buffer, pH 6.0, was used instead of Tris buffer. After incubation for 10 min at room temperature, internal protein marker, human IgG (0.6 mg), was added, and the mixtures were centrifuged for 30 min at 5,000 × g to remove precipitates. The clear supernatants were layered on 5-ml glycerol gradients (5 to 25%, vol/vol) in 50 mM potassium phosphate (pH 6.0), 0.1 M KCl, 0.1% Triton X-100, 10 mM β-mercaptoethanol, and 20% ethylene glycol. Gradients were centrifuged for 23 h at 49,000 rpm in an SW 50.1 rotor and fractionated. DNA polymerase activity and trp-tRNA binding were analyzed as described in the text, and the location of IgG protein marker was determined by assaying the protein content in the fractions after trichloroacetic acid precipitation, according to Lowry et al. (11). (A) wt PR-A virions, 340 μg; (b) purified AMV reverse transcriptase, 100 enzyme units.*

and gel filtration columns under conditions which minimized deacylation of aminoacyl-tRNA, namely, low-pH phosphate buffer.

In all experiments described in this communication, saturating amounts of [^{3}H]trp-tRNA were added, and therefore only 15% of [^{3}H]trp-tRNA bound to the enzyme (Fig. 2A). Under conditions of such tRNA molar excess, any reduction of the enzyme-binding activity should be detectable, thus enabling us to measure binding activity per a given amount of virus. When an excess of virus, rather than tRNA, was used in the binding reactions, as much as 35% of [^{3}H]trp-tRNA bound to the enzyme in crude virus extracts (results not shown). A similar percentage of binding has been previously reported, using a purified preparation of [^{32}P]-tRNA^{trp} (16). No structural difference between bound and unbound fractions has as yet been detected, and it could be that conformational isomers of tRNA^{trp} explain the two fractions.

trp-tRNA binding in LA 335 and LA 336 mutants. Temperature-sensitive mutants LA 335 and LA 336 have been shown to be early replication-defective mutants which contain heat-labile reverse transcriptase (6, 10, 12, 25). The enzyme in these mutants is defective for both DNA polymerase and RNase H (12, 24, 25). The results shown in Table 1 demonstrate that for both mutants, DNA polymerase activity, measured with poly(A)·oligo(dT) template, is heat labile as compared to the wild-type viruses RSV PR-C and ASV B77. Thus, in agreement with the results of Verma et al. (25), preincubation at 45°C resulted in 50% inactivation of the polymerase activity with detergent-disrupted virions of LA 335 and LA 336 after 4 and 2 min, respectively.

Since the specific binding of the primer tRNA^{trp} represents yet another activity of the enzyme probably needed for initiation of DNA synthesis on the viral RNA template, it was of interest to compare the heat lability of the binding site to that of the polymerase site. [^{3}H]trp-tRNA binding activity was estimated, after preincubation of disrupted virions at 45°C for 30 min, by gel filtration of the complex formed. In the control reactions, reverse transcriptase of wt PR-C, LA 335, and LA 336 was shown to be active in binding, as is evident by the co-chro-

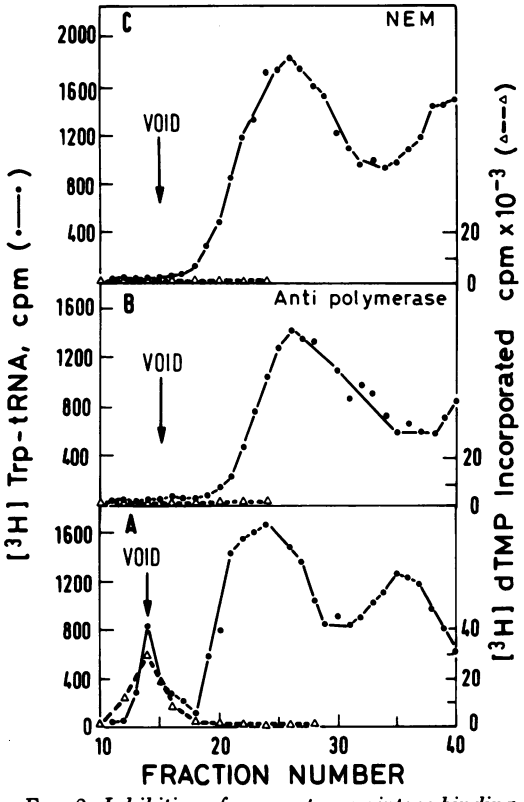

FIG. 2. *Inhibition of reverse transcriptase binding activity in disrupted virions. Virions of wt PR-A (340 μg) were preincubated in binding reaction mixtures, with 0.1 mM dithiothreitol: (A) without additions; (B) with rat IgG monospecific for AMV reverse transcriptase (12 μg); (C) with 2 mM N-ethylmaleimide. After 20 min, dithiothreitol (10 mM) was added to destroy excess NEM, followed by addition of [3H]trp-tRNA (16,000 cpm). The mixtures were chromatographed after an additional 10 min on Sephadex G-100 columns as described in the text.*

matography of [3H]trp-tRNA and the DNA polymerase activity on Sephadex columns (Fig. 3A, C, and E). Preincubation at high temperature hardly affected the wt PR-C activities, whereas both trp-tRNA binding and DNA polymerase activities in mutants LA 335 and LA 336 were completely inactivated (Fig. 3B, D, and F).

Heat sensitivity of DNA polymerase and tRNA binding activities with LA 338, LA 343, and LA 672. The three temperature-sensitive mutants LA 338, LA 343, and LA 672 exhibit a common property; all carry late replication defects which determine that virions produced at the nonpermissive temperature are noninfectious (3, 27). Other important differences emerge between LA 338 and LA 343, on the one side, and LA 672 on the other. LA 338 and LA 343 clearly carry several genetic defects

TABLE 1. *Inactivation rates of temperature-sensitive ASV DNA polymerases*

Virus	Half-life (min) for DNA polymerase activity after preincubation at 45°C[a]
wt PR-C	80
LA 335	4
LA 338/35°C	40
LA 338/42°C	60
wt B77	60
LA 336	2

[a] Mixtures (100 μl) contained 50 mM Tris-hydrochloride, pH 8.3, 6 mM $MgCl_2$, 10 μg of bovine serum albumin, 0.02% Triton X-100, 5 mM dithiothreitol, and virions as follows: B77 and wt PR-C, 32 enzyme units; LA 336, 25.4 enzyme units; LA 338 grown at 35 or 42°C, 33 enzyme units; LA 335, 30 enzyme units. The mixtures were incubated at 45°C, and 10-μl samples were taken into ice. Residual DNA polymerase activity was assayed by adding 90 μl of assay mixture as described in the text.

(2, 9), one of which affects transformation independent of virus replication. The other is responsible for, in addition to the late defect in replication, an early defect, first described by Wyke and Linial (27) as a transient essential function for the establishment of transformation. Recent experiments with LA 338 (Mölling and Friis, manuscript in preparation) indicate that this early defect, which affects replication, is indeed only a separate phenotype caused by a reverse transcriptase lesion, perhaps the same which determines the late defect. Hence, for LA 338, a reverse transcriptase lesion probably causes both an early and a late defective phenotype. LA 338 harvested at 35°C also exhibited in our experiments a significantly reduced half-life compared with wt PR-C after incubation at 45°C. LA 672 apparently has only a single genetic defect, that affecting reverse transcriptase (4). Most interesting with LA 672 is the fact that the reverse transcriptase defect results only in a late defective phenotype; hence, cells infected with stocks of LA 672 prepared at the permissive temperature do not exhibit any temperature sensitivity in the initial establishment of infection. The noninfectious particles of these mutants have the same structural properties as infectious virus, and all the major structural proteins are present in normal amounts (4). We have studied two problems with these mutants. (i) Does the reverse transcriptase contain a temperature-sensitive lesion which effects the initiation of DNA synthesis as measured by the tRNA primer binding activity, and (ii) is the reduced DNA polymerase activity in virions produced at the nonpermissive temperature (42°C) due to an inactive enzyme or to a reduced amount of active reverse

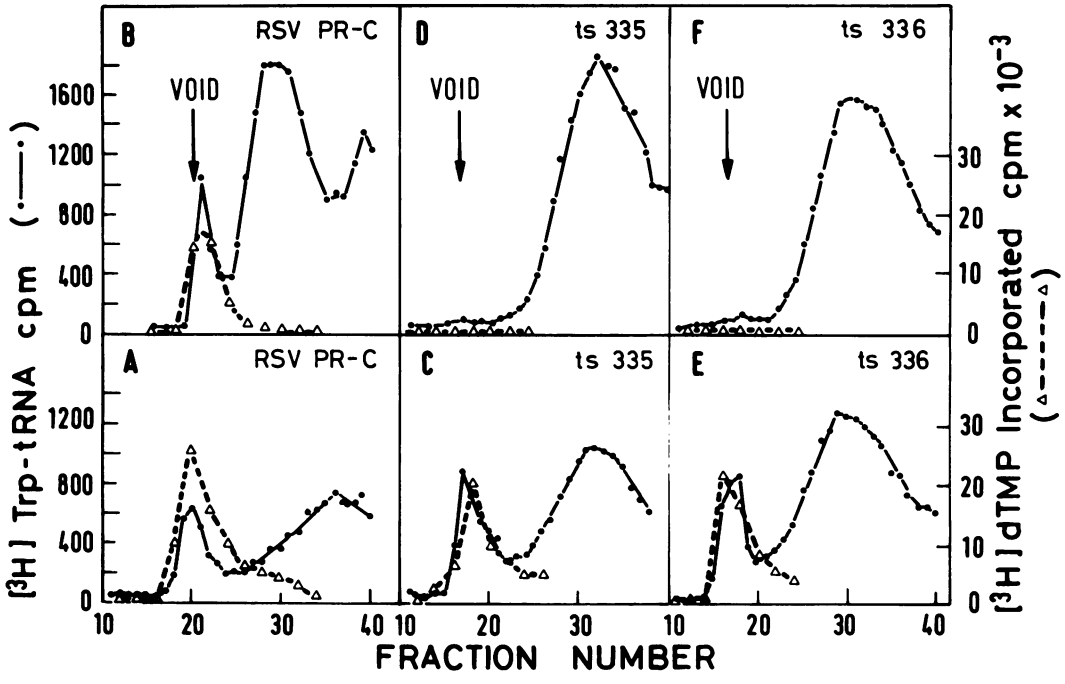

FIG. 3. *Inactivation of trp-tRNA binding activity in LA 335 and LA 336. (B, D, and F) Virions were preincubated in binding mixture at high temperature (45°C) for 30 min, and [^{3}H]trp-tRNA was then added. (A, C, and E) Binding was conducted without preincubation as described in the text. (A and B) wt PR-C (330 μg); (C and D) LA 335 (325 μg); (E and F) LA 336 (330 μg).*

transcriptase assembled into virions?

The observation of Verma et al. (25) that in vivo proviral DNA synthesis is reduced when infection with LA 338 is carried out at the nonpermissive temperature, even though in vitro DNA polymerase activity is heat stable, prompted us to investigate whether the initiation of DNA synthesis, as measured by the trp-tRNA binding activity, is temperature sensitive in this virus. The results shown in Fig. 4 indicate that the tRNA binding activity of reverse transcriptase from LA 338 virions, grown at either the permissive (35°C) or the nonpermissive temperature (42°C), is not thermolabile. Disrupted virions were preincubated at high temperature (45°C) or left at 4°C for 30 min, followed by addition of [^{3}H]trp-tRNA and analysis by gel filtration. The enzyme in LA 338 grown at the permissive temperature and later preincubated at 45°C can bind as much [^{3}H]trp-tRNA as the same enzyme which was kept at 4°C (Fig. 4A and B). Similarly, trp-tRNA binding to noninfectious virions, produced at 42°C, is heat stable, and preincubation at 45°C for 30 min did not affect the binding activity of that reverse transcriptase (Fig. 4C and D). Preincubation of disrupted virions at 45°C resulted, however, in a small decrease in the total amount of DNA polymerase eluted from the Sephadex column (see also Table 1).

To investigate the biochemical nature of the late replication lesion in LA 338, we quantitated the DNA polymerase and tRNA binding activities in virions made at both 35 and 42°C. A given amount of virions produced at 35°C bind fourfold more trp-tRNA as compared with virions made at the nonpermissive temperature (42°C) (Fig. 4A and C; Table 2). A similarly enhanced (three- to fourfold) specific activity of DNA polymerase is noticed in virions produced at 35°C as compared with those made at 42°C. The total DNA polymerase and [^{3}H]trp-tRNA binding activities eluted from the columns (Table 2) were calculated from the results shown in Fig. 4A and C by integrating the area under the void volume peaks.

LA 343 was also analyzed for its reverse transcriptase properties (Fig. 5). The virions produced at the nonpermissive temperature (42°C) show a fourfold-lower specific activity for trp-tRNA binding compared with virions grown at 35°C, and the DNA polymerase specific activity in the noninfectious virus was reduced by a factor of 2.5. Wild-type PR-C produced at either 42 or 35°C are both infectious and have specific activities for tRNA binding and DNA polymer-

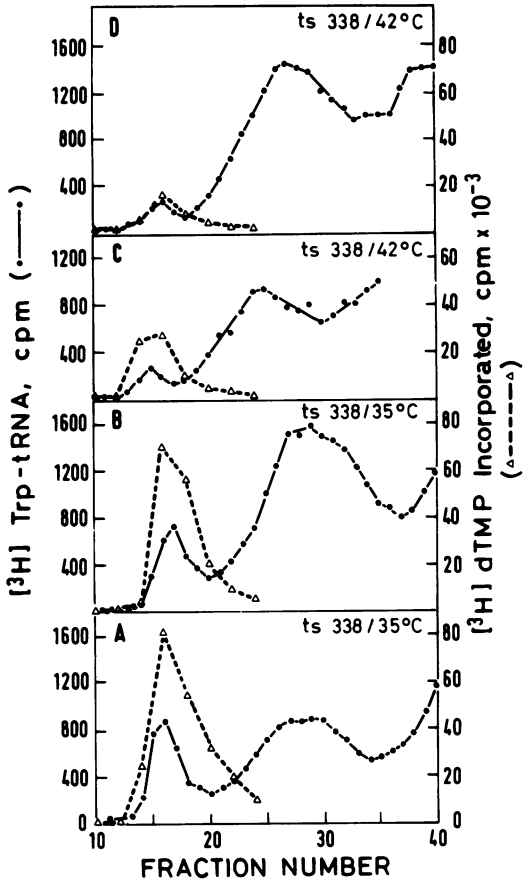

FIG. 4. *trp-tRNA binding and DNA polymerase activities in LA 338. Binding reactions were conducted as described in the text. (A) LA 338, grown at 35°C (330 μg), and (B) the same virions were preincubated in the binding mixture for 30 min at 45°C before [^{3}H]trp-tRNA was added. (C) LA 338 grown at 42°C (330 μg) and (D) the same virions were preincubated, for 30 min at 45°C, before [^{3}H]trp-tRNA was added.*

ase similar to the mutant virus LA 343 produced at 35°C (Fig. 5A). The results of these two independent assays for tRNA binding and DNA polymerase suggest that the late temperature-sensitive lesions in LA 338 and LA 343 affect the amounts of active reverse transcriptase in virions produced at the nonpermissive temperature.

LA 672 also appears to be defective in a late stage of replication in the synthesis or assembly of a functional reverse transcriptase (4). We have compared the tRNA binding as well as the DNA polymerase activities in infectious virions produced at 35°C and noninfectious virions made at 42°C. Such a study gives us information as to whether the noninfectious virions of LA 672

TABLE 2. *Summary of reverse transcriptase activities and phenotypes of LA 672, LA 338, and LA 343 propagated at permissive and nonpermissive temperatures*

Virus	DNA polymerase[a] (enzyme units)	[^{3}H]trp-tRNA binding[b] (pmol)	Relative amt of antigen[c]	Efficiency of replication[d]
LA 338/35°C	16	0.64	0.36	1
LA 338/42°C	5	0.15	0.19	0.08
LA 343/35°C	23	0.94	0.5	1
LA 343/42°C	9	0.22	0.14	0.07
LA 672/35°C	4.0	0.55	0.45	1
LA 672/42°C	0.7	0.15	0.09	0.0025

[a] Total DNA polymerase activity eluted from Sephadex G-100 columns was calculated by summing the enzyme activity in the void volume (usually fractions 13 to 18 in Fig. 4, 5, and 6).

[b] [^{3}H]trp-tRNA bound is the sum of radioactivity co-chromatographed with the enzyme in the void volume (usually fractions 13 to 18). Virion protein input was around 330 μg (see legends to Fig. 4, 5, and 6).

[c] Relative amount of reverse transcriptase antigen in virions was derived from amount (micrograms) of viral protein needed to restore 20 or 30% of the DNA polymerase activity in the immunoassay (Fig. 7). At 20 to 30% of enzyme activity the competition immunoassay is linear, and therefore the amount of competing virus needed to restore this activity is inversely linear to the virus content of reverse transcriptase antigen. The amount of antigen in wt PR-A or PR-C was adjusted to 1 unit, and the relative amounts of *ts* mutants were accordingly corrected.

[d] Efficiency of mutant replication was calculated from the titers of infectious virus released to the medium at 35 and 42°C. The focus assay was carried out under standard conditions (2, 3).

contain enzyme protein which has lost DNA polymerase activity but still retains its tRNA binding activity. The results illustrated in Fig. 6A indicate that infectious virus produced at the permissive temperature (35°C) contains a high tRNA binding activity. An equal amount (micrograms of protein) of noninfectious virions (produced at 42°C) binds threefold less trp-tRNA but exhibits a sixfold reduction in DNA polymerase activity as compared with LA 672 produced at 35°C (Fig. 6; Table 2). The standard LA 672 virions used in this study were harvested every 24 h and then purified. To exclude the possibility that the virions produced at 42°C were inactivated due to the high temperature after release from cells, we also investigated the reverse transcriptase in rapidly harvested (3 h) virus. A rapidly harvested virus grown at 42°C (Fig. 6C) behaves similarly to the 24-h-harvested virus (Fig. 6B), and its polymerase and tRNA binding activities are both reduced compared

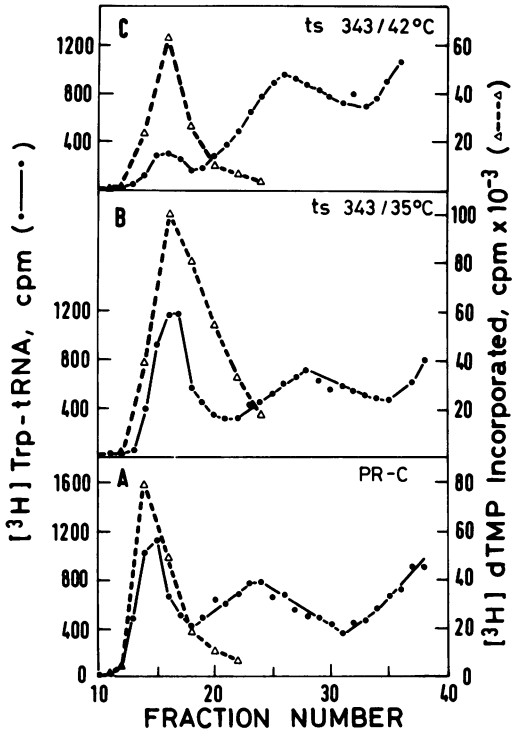

FIG. 5. *trp-tRNA binding and DNA polymerase activities in LA 343. Binding of [^{3}H]trp-tRNA was carried out as described in the text. (A) PR-C grown at 42°C (300 μg); (B) LA 343 grown at 35°C (325 μg); (C) LA 343 grown at 42°C (327 μg).*

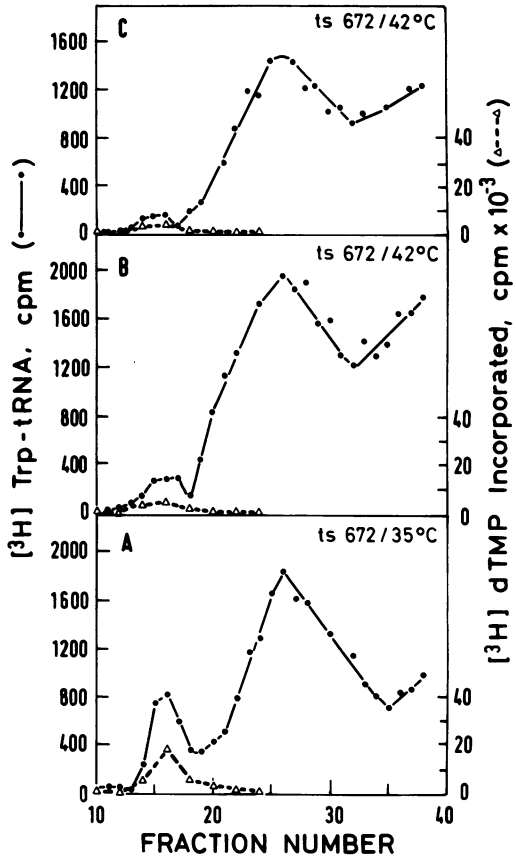

FIG. 6. *trp-tRNA binding and DNA polymerase activities in LA 672. Conditions for binding and DNA polymerase activity are described in the text. (A) LA 672, grown at 35°C, harvested at 24-h intervals (345 μg); (B) LA 672, grown at 42°C, harvested at 24-h intervals (345 μg); (C) LA 672, grown at 42°C, harvested every 3 h (360 μg).*

with virus grown at the permissive temperature, 35°C (Fig. 6A). All of these results indicate that qualitatively the lesion in LA 672 is similar to the late temperature-sensitive function in LA 338 and LA 343.

Quantitation of reverse transcriptase antigen in temperature-sensitive ASV mutants. The finding of reduced DNA polymerase and trp-tRNA binding activities in ASV mutants LA 338, LA 343, and LA 672 grown at the nonpermissive temperature could be explained in either of two ways: (i) packaging of enzyme into virions is a temperature-sensitive process, and virions produced at 42°C contain less reverse transcriptase protein; or (ii) enzyme synthesized in the cells at high temperature is defective, and, as a result, virions contain normal amounts of enzyme protein with low enzymatic activities. To differentiate between these two possibilities, we measured the relative amounts of reverse transcriptase antigen in virions by using a competition immunoassay (15). Detergent-disrupted virions are reacted with NEM, which inhibits in a nonreversible fashion the DNA polymerase activity but does not affect the antigenic properties of the reverse transcriptase, and excess NEM is destroyed by dithiothreitol. Increasing amounts of the NEM-treated virions are then reacted with a limited amount of anti-reverse transcriptase IgG. The ability of the mutant viruses to absorb the monospecific IgG can then be estimated by adding purified active reverse transcriptase to react with the free IgG, and finally the residual reverse transcriptase activity is estimated with poly(A) oligo(dT) as template primer for DNA synthesis (Fig. 7). Virions which contain a large amount of reverse transcriptase antigen will absorb more antibodies, and as a result less of the purified reverse transcriptase will be inactivated in the final reaction. The neutralization inhibition method used here proved to be as accurate as the standard competition radioimmunoassay technique

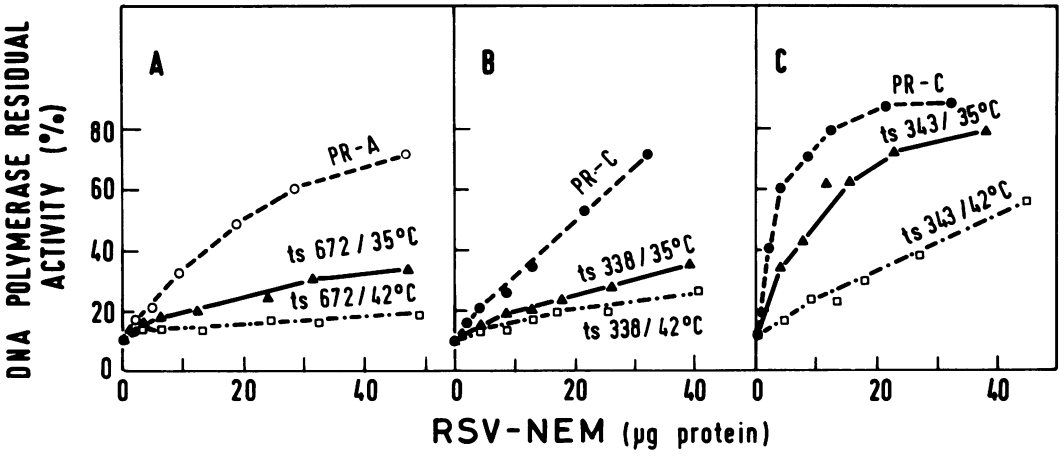

FIG. 7. *Neutralization inhibition of DNA polymerase in RSV ts mutants. The reactions were conducted in four steps. (i) DNA polymerase activity in virions was inactivated by incubation with 2 mM NEM, 50 mM Tris-hydrochloride (pH 7.5), 1.0 mg of bovine serum albumin per ml, and 1.0% Triton X-100. After 15 min at 37°C, the tubes were transferred to 4°C and dithiothreitol (7 mM) was added to destroy excess NEM. (ii) Increasing amounts of virus-NEM were reacted for 20 min at 37°C with anti-reverse transcriptase IgG (3 μg) in mixtures (45 μl) containing 10 mM Tris-hydrochloride (pH 8.0), 100 mM KCl, 0.5 mg of bovine serum albumin per ml, 10% glycerol, and 0.1% Triton X-100. (iii) Active purified AMV reverse transcriptase, 1.5 units/5 μl, was added, and incubation was continued for another 20 min at 37°C. (iv) The residual DNA polymerase activity was determined by adding a mixture (50 μl) of all the components needed for assaying DNA polymerase activity (see text), and the incubation time was 20 min. Under the above conditions, the enzymatic activity was linear for at least 40 min. The percentage of residual DNA polymerase activity was calculated by comparison with control reactions in duplicate tubes that contained no immune IgG (taken as 100%).*

for reverse transcriptase (15). Thus, to restore 20% of the probe DNA polymerase activity, 55 μg of LA 672 virions grown at 42°C, 11 μg of virions grown at 35°C, and 5 μg of wt PR-A virus were needed (Fig. 7A). These results indicate that LA 672 virus grown at 42°C contains about fivefold less enzyme protein as compared with the same virus grown at the permissive temperature (35°C). LA 338 and LA 343 mutants show a similar lack, and virions produced at the nonpermissive temperature contain two- to fourfold less reverse transcriptase as compared with virus made at 35°C and some sixfold less enzyme than the wt PR-C (Fig. 7B and C; Table 2).

All of these results indicate that the three conditional mutants analyzed, LA 672, LA 338, and LA 343, contain reduced amounts of reverse transcriptase protein per total virus proteins when harvested at the nonpermissive temperature. It is interesting to note, however, that even the infectious virus mutants produced at the permissive temperature always contain less enzyme protein than the wild-type parents. Whereas the reduced incorporation of reverse transcriptase into virions can explain lower DNA polymerase and trp-tRNA binding for LA 338 and LA 343 grown at 42°C, LA 672 grown at 42°C probably incorporates significant amounts of damaged enzyme, since the trp-tRNA binding and DNA polymerase activities for these virions grown at 42°C are not parallel.

DISCUSSION

In the present study we describe the interaction between the tRNA primer for DNA synthesis and the reverse transcriptase of five temperature-sensitive mutants of ASV. The finding that trp-tRNA binding activity in LA 335 and LA 336 is thermolabile provides the best proof that this function is virus coded like the DNA polymerase and the RNase H activities (24, 25). The active sites for these three activities are apparently a part of the same gene product since they are coordinately affected by single mutations. Biochemical studies, however, show only little overlapping of the three active sites. Thus, each of the three activities can be separately inhibited by different chemical reagents (Gorecki and Panet, manuscript in preparation).

Our findings with the late replication-defective mutants indicate that in terms of DNA polymerase and trp-tRNA binding, reverse transcriptase is either not at all (LA 343 and LA 672) or only slightly (LA 338) thermolabile. In fact, the lesions responsible for the phenotype ob-

served need not necessarily be in the genetic element coding for the viral reverse transcriptase (*pol*) but could be located in an unknown function essential for correct virus assembly. More probably, however, defective reverse transcriptase molecules are themselves impeded in some kind of self-assembly process, and the actual defects are caused by lesions in *pol*. The findings presented in Fig. 7 and Table 2, in any case, go a long way in explaining the lack of infectivity of virus isolated from cultures maintained at 42°C.

Certain complications remain, however, if the late defective mutants are to be explained entirely on the basis of lack of reverse transcriptase incorporation. First, there is the interesting fact that whereas specific activity of DNA polymerase observed with LA 672 isolated from cultures at 42°C was reduced sixfold (and more in some experiments), trp-tRNA binding by the same virus preparations was less reduced. This finding was consistently observed in repeated experiments. Hence, for LA 672, the fractional complement of reverse transcriptase, which does incorporate into virions grown at 42°C, seems to have retained more trp-tRNA binding activity than DNA polymerase activity. This would point quite definitely to a *pol* lesion. Furthermore, analyses of recombinants made between the avian leukosis virus, RAV-6, and LA 672 (Joho, Stoll, Friis, and Weissmann, unpublished observations) showed that T_1 RNase-resistant oligonucleotides directly associated with the temperature-sensitive phenotype were those which mapped in the region defined as coding for *pol*.

Likewise, LA 338 can be considered a likely mutant in *pol*. A significantly shorter half-life for inactivation of the reverse transcriptase was noted with this mutant (Table 1), regardless of whether virus preparations had been grown at 35 or 42°C. Since this mutant shows an additional very leaky, early, transient defect (27), it may well be speculated that there is a slight (leaky) early *pol*-related defective phenotype. Evidence for this early defect can be found in the thermolability referred to above and in similar recent experiments showing temperature-sensitive behavior of LA 338 in terms of both DNA polymerase and RNase H activities (Mölling and Friis, manuscript in preparation).

The amount of enzyme in noninfectious virions of LA 338, LA 343, and LA 672 produced at the nonpermissive temperature is three- to sixfold lower than in the same viruses produced at the permissive temperature. The amount of reverse transcriptase per wt ASV virion was calculated by radioimmunoassay to be 30 to 70 molecules (15). A sixfold reduction would leave 5 to 12 enzyme molecules in mutant virions produced at the nonpermissive temperature. It is interesting that the reduction in infectivity of mutant virus preparations grown at the nonpermissive temperature is 100- to 500-fold. This disparity between loss of enzyme and reduced infectivity may be seen as evidence that infectivity requires more than just a few molecules of reverse transcriptase per virion, or it may be interpreted to suggest that, indeed, LA 338, LA 343, and LA 672 incorporate not just reduced amounts of reverse transcriptase, but also contain molecules of lower specific activity.

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LITERATURE CITED

1. **Baltimore, D.** 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. Nature (London) **226**:1209–1211.
2. **Blair, D. G., W. S. Mason, E. Hunter, and P. K. Vogt.** 1976. Temperature sensitive mutants of avian sarcoma viruses: genetic recombination between multiple or coordinate mutants and avian leukosis viruses. Virology **75**:48–59.
3. **Friis, R. R., and E. Hunter.** 1973. A temperature sensitive mutant of Rous sarcoma virus that is defective for replication. Virology **53**:479–483.
4. **Friis, R. R., W. S. Mason, Y. C. Chen, and M. S. Halpern.** 1975. A replication defective mutant of Rous sarcoma virus which fails to make a functional reverse transcriptase. Virology **64**:49–62.
5. **Friis, R. R., H. Ogura, H. Gelderblom, and M. S. Halpern.** 1976. The defective maturation of viral progeny with a temperature sensitive mutant of avian sarcoma virus. Virology **73**:259–272.
6. **Friis, R. R., K. Toyoshima, and P. K. Vogt.** 1971. Conditional lethal mutants of avian sarcoma viruses. I. Physiology of ts 75 and ts 149. Virology **43**:375–389.
7. **Grandgenett, D. P., A. C. Vora, and A. J. Faras.** 1976. Different states of avian myeloblastosis virus DNA polymerase and their binding capacity to primer $tRNA^{trp}$. Virology **75**:26–32.
8. **Hanafusa, H., D. Baltimore, D. Smoler, K. F. Watson, A. Yaniv, and S. Spiegelman.** 1972. Absence of polymerase protein in virions of alpha type Rous sarcoma virus. Science **177**:1188–1191.
9. **Hunter, E., and P. K. Vogt.** 1976. Temperature sensitive mutants of avian sarcoma viruses: genetic recombination with wild type sarcoma virus and physiological analysis of multiple mutants. Virology **69**:23–34.
10. **Linial, M., and W. S. Mason.** 1973. Characterization of two conditional early mutants of Rous sarcoma virus. Virology **53**:258–273.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. **Mason, W. S., R. R. Friis, M. Linial, and P. K. Vogt.** 1974. Determination of the defective function in two mutants of Rous sarcoma virus. Virology **61**:559–574.
13. **Moelling, K., D. P. Bolognesi, H. Bauer, W. Busen, H. W. Plassmann, and P. Hausen.** 1971. Association

of viral reverse transcriptase with an enzyme degrading the RNA moiety of RNA-DNA hybrids. Nature (London) New Biol. **234**:240–243.

14. **Nishimura, S., and B. I. Weinstein.** 1969. Fractionation of rat liver tRNA. Isolation of tyrosine, valine, serine and phenylalanine tRNA and their coding properties. Biochemistry **8**:832–842.
15. **Panet, A., D. Baltimore, and T. Hanafusa.** 1975. Quantitation of avian RNA tumor virus reverse transcriptase by radioimmunoassay. J. Virol. **16**:146–152.
16. **Panet, A., W. H. Haseltine, D. Baltimore, G. Peters, F. Harada, and J. E. Dahlberg.** 1975. Specific binding of tryptophan-transfer RNA to avian myeloblastosis virus RNA-dependent DNA polymerase (reverse transcriptase). Proc. Natl. Acad. Sci. U.S.A. **72**:2535–2539.
17. **Panet, A., I. M. Verma, and D. Baltimore.** 1974. Role of the subunits of the avian RNA tumor virus reverse transcriptase. Cold Spring Harbor Symp. Quant. Biol. **39**:919–923.
18. **Rogg, H., W. Wehrli, and M. Staehelin.** 1969. Isolation of mammalian transfer RNA. Biochim. Biophys. Acta **195**:13–15.
19. **Taylor, J. M.** 1977. An analysis of the role of tRNA species as primers for the transcription into DNA of RNA tumor virus genomes. Biochim. Biophys. Acta **473**:57–71.
20. **Temin, H. M., and S. Mizutani.** 1970. RNA dependent DNA polymerase in virions of Rous sarcoma virus. Nature (London) **226**:1211–1213.
21. **Toyoshima, K., and P. K. Vogt.** 1969. Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions. Virology **38**:414–426.
22. **Toyoshima, K., and P. K. Vogt.** 1969. Temperature sensitive mutants of an avian sarcoma virus. Virology **39**:930–931.
23. **Verma, I. M.** 1977. The reverse transcriptase. Biochim. Biophys. Acta **473**:1–38.
24. **Verma, I. M., W. S. Mason, S. D. Drost, and D. Baltimore.** 1974. DNA polymerase activity from two temperature-sensitive mutants of Rous sarcoma virus is thermolabile. Nature (London) **251**:27–31.
25. **Verma, I. M., H. E. Varmus, and E. Hunter.** 1976. Characterization of early temperature sensitive mutants of avian sarcoma viruses: biological properties, thermolability of reverse transcriptase in vitro and synthesis of viral DNA in infected cells. Virology **74**:16–29.
26. **Watson, K. F., R. Nowinski, A. Yaniv, and S. Spiegelman.** 1972. Serological analysis of the deoxyribonucleic acid polymerase of avian oncornaviruses. I. Preparation and characterization of monospecific antiserum with purified deoxyribonucleic acid polymerase. J. Virol. **10**:951–958.
27. **Wyke, J. A., and M. Linial.** 1973. Temperature-sensitive avian sarcoma viruses: a physiological comparison of twenty mutants. Virology **53**:152–161.