Volume 2 number 12 December 1975 Nucleic Acids Research

E.coli tRNAs as inhibitors of viral reverse transcription in vitro.

Liebe F.Cavalieri and Izumi Yamaura

Sloan-Kettering Institute for Cancer Research, Donald S.Walker Laboratory, 145 Boston Post Road, Rye, NY 10580, USA

Received 7 November 1975

ABSTRACT

Reverse transcription of 70S AMV RNA by AMV reverse transcriptase has been studied in the presence of E. coli tRNAs. We have shown that inhibition of DNA synthesis occurs and that the tRNAs bind to the enzyme and not to the 70S RNA. The results have implications for the control of reverse transcription *in vivo*.

INTRODUCTION

In this study we show that *E. coli* tRNAs inhibit AMV reverse transcriptase *in vitro* using 70S AMV RNA as the primer-template. Furthermore, we show that the tRNAs bind to the reverse transcriptase and not to the 70S RNA. The mechanism of the interaction of reverse transcriptase with viral RNA is unknown but $tRNA^{Trp}$ has been shown to be an initiator of DNA synthesis on AMV RNA *in vitro* (1-8). The roles of other tRNAs found within virions, some of which are attached to the 70S RNA, are unknown. In investigations designed to learn the answers to some of these questions we have examined the secondary structure of AMV RNA (9) and also the kinetics of reverse transcription using a reconstructed system composed of the purified AMV 70S RNA and reverse transcriptase (10). The latter study shows that reverse transcription *in vitro* depends in an important manner on the physical state of the polymerase, which in turn is determined by the ionic environment of the reaction mixture: oligomers of reverse transcriptase, which exist at low ionic strength, behave differently toward AMV RNA than do monomers, which exist predominantly at high ionic strength (10). We have now turned to an examination of the effects

of E. coli tRNAs in an attempt to learn their role(s) in the in vitro reaction. The results have been sufficiently encouraging that we are now investigating the possible inhibitory effects of tRNAs from bacterial and various eukaryotic sources on transformation of cells by oncornaviruses and on virus production (11).

MATERIALS AND METHODS

Deoxynucleoside-5'-triphosphates and primer-templates.

The unlabeled 5'-triphosphates were purchased from P-L Laboratories. The labeled precursors were purchased from New England Nuclear Corp. The specific activities were about 15 Ci/mmole, as purchased. The primer-templates poly(rA)dT_{10} were prepared from the components by annealing for 3 minutes at 55^{0} followed by slow cooling to room temperature; the solvent was 0.05 M Tris, pH 7.8, and the total concentration of homopolymer + oligomer was 75 µg/ml in each case. The specific activity of the labeled precursors in the assay procedures was 300 cpm/pmole when using the homopolymers and 1200 cpm/pmole when using AMV RNA.

Reverse Transcriptase.

This enzyme was supplied by Life Sciences Incorporated, St. Petersberg, Florida under contract NO1CP33291, Virus Cancer Program, National Cancer Institute. It was prepared according to the procedure of Kacian et al. (12) and is the material obtained from the phosphocellulose column; its specific activity was about 1300 units/mg of protein but decreased to 700 units (preparation 1) after one year. We have purified this material to a specific activity of about 10,000 units/mg protein by passing it through a second phosphocellulose column. A similar purification carried out at the Life Sciences Laboratories has yielded a preparation of 32,000 units/mg protein (preparation 2); the latter was supplied by Dr. J. Beard. Most of the assays described in this report were carried out with the less pure enzyme. However, replicate runs of the tRNA inhibition reactions using the purer enzyme

yielded similar results. Typical results are given in Table I. Therefore we feel safe in concluding that any irrelevant proteins present in the preparations have not altered our results in any important way. We have also examined the effects of enzyme purity on the nature of the kinetics of DNA synthesis, using AMV RNA as the primer-template, in another paper (10) and found that those results were also unaltered by the degree of enzyme purity.

The unit of activity is defined as that amount of enzyme which catalyzes the incorporation of one nmole of TMP in the presence of poly(rA)dT_{10} in 10 minutes at 37° C under standard assay conditions (12).

Avian Myeloblastosis Virus (AMV).

Purified virus pellets were obtained from Life Sciences Incorp. The time elapsed from bleeding the chickens to pelleting of the virus was six hours; therefore nicking of the viral RNA within the virion was minimal. This was shown by analytical centrifugation of the heated 70S RNA; the results are given in the next section.

TABLE I

EFFECT OF ENZYME PURITY ON INHIBITION BY VARIOUS tRNAs

		Percent Activity Remaining	
Ratio	70S:tRNA	Preparation 1	Preparation 2
E. coli tRNA	1:00	100	100
	1:0.1	76	72
	1:0.25	60	62
E. coli $tRNA^{Phe}$	1:0.0	100	100
	1:0.1	81	73
	1:0.25	67	56
Yeast tRNA	1:0.0	100	100
	1:0.1	89	67
	1:0.25	52	35

Assays were carried out and processed as described in Materials and Methods. Each assay contained 1.4 µg of 70S RNA and an amount of tRNA corresponding to the ratio given in the first column. Preparation 1 contained 1.4 units/assay and preparation 2, 6.1 units/assay.

AMV RNA.

Viral RNA was extracted according to the procedure of Kacian et al. (13). The RNA corresponding to the 70S peak in the 10-30% glycerol gradient was pooled and either used directly or precipitated with 95% ethanol - 0.3 M NaCl. Analysis in the Model E analytical ultracentrifuge showed various RNA preparations to have $S_{20,w}$ values in the range 55-70 S. Heat-denatured RNA (100^{o}, 3 min in 0.01 M Tris - 0.1 M NaCl - 1 mM EDTA) showed one sedimenting boundary in the 20-30 $S_{20,w}$ range. In some cases when the RNA had been kept frozen at -20^{o} for about one month there was a higher background of optical density, indicating the presence of degraded, non-sedimenting material.

tRNAs.

E. coli K-12 MO tRNA, $tRNA_f^{Met}$ and $tRNA^{Phe}$ were purchased from Miles Laboratories, Kankakee, Illinois. These are high purity preparations obtained by reversed-phase chromatography. ^{3}H-tRNA (E. coli K-12 MO) was purchased from Miles and was also isolated by reversed-phase chromatography; its specific activity is 43 µCi/50 µg. About 24,000 cpm (= 0.04 µg) were used in each assay. This material contained no detectable ribosomal or 5S RNAs and no DNA nor protein.

Assay Procedures.

The standard assay for 70S RNA contains (in 0.10 ml): 0.030 M Tris pH 7.8, 0.005 M $MgCl_2$, 0.12 M NaCl, 2.0 mM EDTA, 1 mM mercoptoethanol, 0.025% BSA, 5.0 µg Actinomycin D, 8 µM each of three unlabeled deoxynucleoside-5'-triphosphates, and 8 µM of either H^3-TTP or H^3-dCTP (1200 cpm/pmole). The concentration of 70S RNA was 1.4 µg/assay. The amount of reverse transcriptase in each assay is given in units in the Figures. The ionic strength of the above solution is 0.165.

The standard assay with poly(rA)dT_{10} contains (in 0.10 ml): 0.028 M Tris, pH 7.8, 0.005 M $MgCl_2$, 0.01 M NaCl, 0.1 mM EDTA, 1 mM mercoptoethanol, 0.025% BSA, 5.0 µg Actinomycin D, 8 µM H^3-TTP (specific activity 300 cpm/pmole)

and 0.4 μg of poly(rA)dT_{10}.

The reaction mixtures were incubated for 30 minutes at 37°. We have shown that the rate of incorporation is linear up to thirty minutes. Therefore the values shown in all figures represent initial rates of reaction. Reactions were initiated by addition of the enzyme to the reaction mixture; they were terminated by addition of 5% (w/v) trichloroacetic acid containing 0.01 M sodium pyrophosphate. Acid-insoluble material was collected by filtration onto Whatman glass filters (GF/B); dried filters were counted in a toluene-base scintillation fluid. Ionic strengths were varied by changing the concentration of the NaCl.

Sedimentation Velocity in Sucrose Gradients.

Sedimentation was carried out in 10-30% (w/v) sucrose solutions containing the assay reaction buffer. Fractions (12 drops) were collected from the top by means of a Buchler Densiflo apparatus. 10 λ of each fraction was assayed for reverse transcriptase by using poly(rA)dT_{10} as the primer-template as described above. When ^{3}H-tRNA was present in the gradient (Figures 3 and 4) the counts contributed by the ^{3}H-tRNA to the reverse transcriptase assay were less than 10% and therefore did not sensibly alter the results. $S_{20,w}$ values were calculated from the tables published by McEwen (14). We have found these calculations to give reliable results based on known standards.

RESULTS

Kinetics of Reverse Transcription.

Figure 1 shows the rate of DNA synthesis as a function of increasing reverse transcriptase concentration. As was reported earlier, the curve exhibits a sigmoidal character, probably due to the presence of inactive sites (10). On adding E. coli tRNAs in a weight ratio of 70S RNA:tRNA of 1:0.1, 1:0.25 and 1:0.5 (Figure 1, curves 2, 3 and 4) the rate of DNA synthesis was markedly decreased. All curves retained their sigmoid nature without change; therefore the added tRNAs do not affect the number of inactive sites.

Binding of tRNA to Reverse Transcriptase.

In order to examine whether the inhibition was due to binding of tRNAs to the 70S RNA or to the reverse transcriptase we carried out the following experiments. Reverse transcriptase was centrifuged in a sucrose velocity density gradient containing our standard assay buffer, in the presence and absence of varying amounts of E. coli tRNAs. The results are shown in Figure 2, where the sedimentation constant increases from 7.2 in the absence of tRNA to 8.0 in the presence of 2.5 μg of $tRNA^{Phe}$. With 0.5 μg of $tRNA^{Phe}$ the S was shifted to 7.9. The original solution (100 λ) which was layered on the gradient contained approximately 0.5 μg of pure reverse transcriptase and the indicated

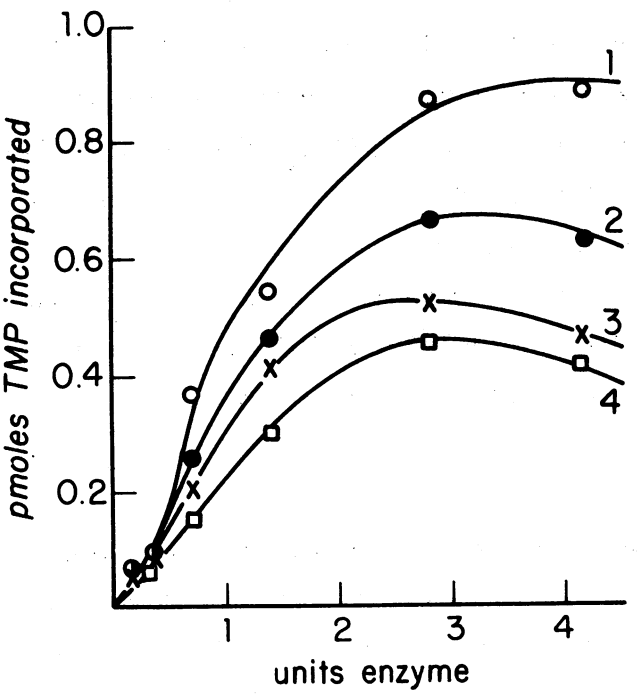

Figure 1. Reverse transcription as a function of enzyme concentration at different E. coli tRNA concentrations. The assays were carried out as described in Materials and Methods. Each assay mixture contained in addition to all of the other components: curve 1, control containing no tRNA, o——o; curve 2, 0.14 μg E. coli tRNA, ●——●; curve 3, 0.35 μg, x——x; curve 4, 0.7 μg, □——□ in each assay tube.

amount of E. coli tRNA^{Phe}. If all of the 0.5 µg of tRNA^{Phe} were bound to the enzyme there would be about 4 or 5 molecules of tRNA/enzyme molecule; this ratio is, of course, a maximum.

As an independent check of the binding of E. coli tRNAs to the reverse transcriptase we used much smaller amounts of mixed E. coli ^{3}H-tRNAs having a high specific activity, and sedimentation was carried out in a phosphate buffer (Fig. 3). In this case the sedimentation constant of the reverse transcriptase was unaffected by the tRNA, but we observed a redistribution of the tRNA

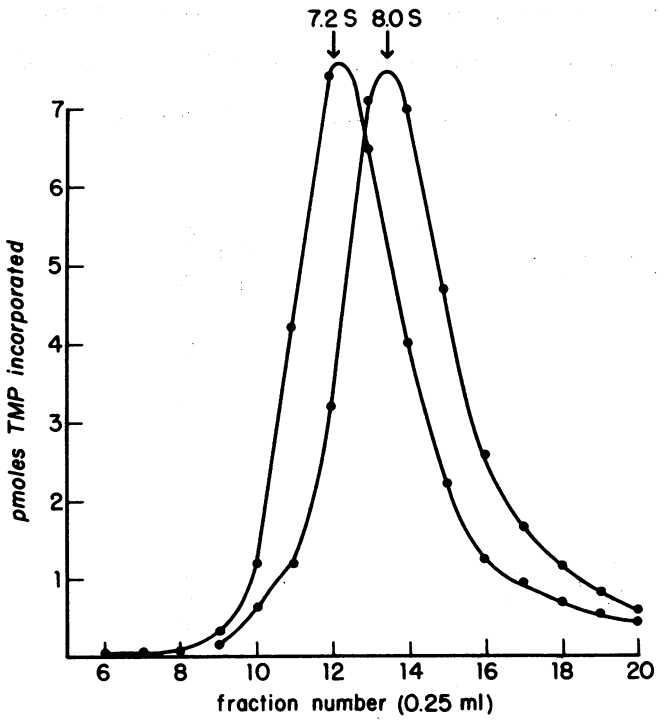

Figure 2. Sedimentation velocity in sucrose density gradients (10-30% w/v). In addition to sucrose the gradient solution contained assay reaction buffer. The curve on the left of the figure represents reverse transcriptase alone. The curve on the right was run in a separate tube containing the same components in addition to 2.5 µg E. coli tRNA^{Phe} in the 100 λ of solution layered on top. In a separate tube (data not shown) the run was carried out with 0.5 µg of tRNA^{Phe}; in this case the sedimentation constant was 7.9 and the curve was omitted to avoid crowding the figure. The runs were carried out in an SW 50.1 rotor for 17 hours at 5^{o} and 45,000 rpm. Assays for reverse transcriptase activity were carried out using poly rAdT$_{10}$, as described above.

tritium counts. The results in Figure 3 show that almost half of the total E. coli ^{3}H-tRNAs were found under the enzyme peak at 7.2 S, indicating that many or most of the tRNAs can bind to the reverse transcriptase. Under these conditions there is probably only one tRNA bound per enzyme molecule. In the presence of higher concentrations of tRNA as discussed above (Figure 2) there must be more than one tRNA bound per enzyme molecule.

When the same experiment was carried out in assay buffer, tRNA was again observed to bind to reverse transcriptase. In the lower panel of Figure 4 we show the sedimentation velocity pattern of E. coli ^{3}H-tRNAs and reverse transcriptase in separate centrifuge tubes. The upper panel of this Figure shows the patterns after the ^{3}H-tRNAs and reverse transcriptase are first mixed and then run in the same centrifuge tube. In this case, although there was no

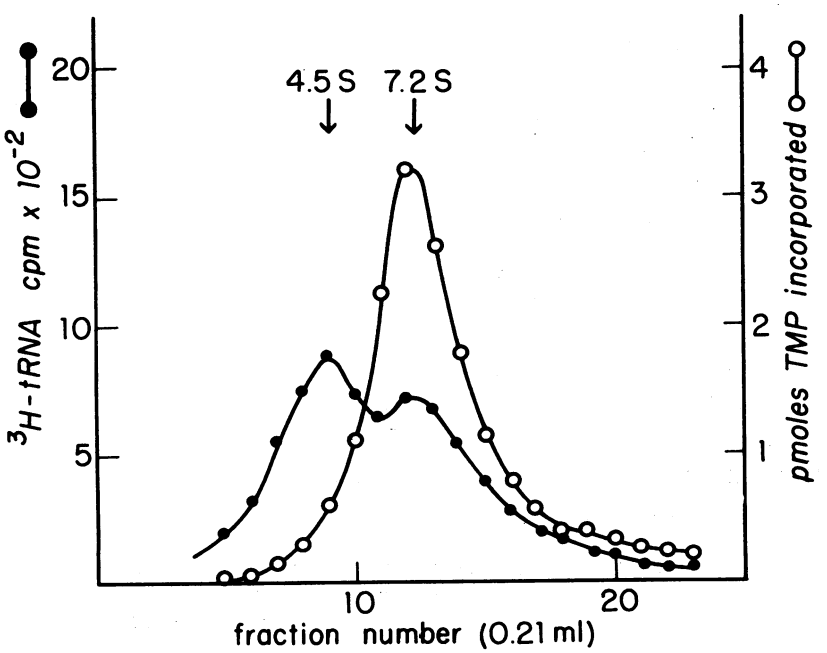

Figure 3. Sedimentation velocity in a sucrose density gradient (10-30% w/v). In this case the buffer in the gradient contained: 0.1 M KPO_4, pH 7.5, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1 mM EDTA and 0.1% Nonidet P-40. The ^{3}H-tRNA and reverse transcriptase were mixed, layered, monitored and run as in Figure 4, upper panel.

discrete ^{3}H-tRNA peak under the reverse transcriptase peak, it is noteworthy that the peak sedimentation constant of the ^{3}H-tRNA is shifted to a higher value, 5.0, and that the band is considerably broadened on the high molecular weight

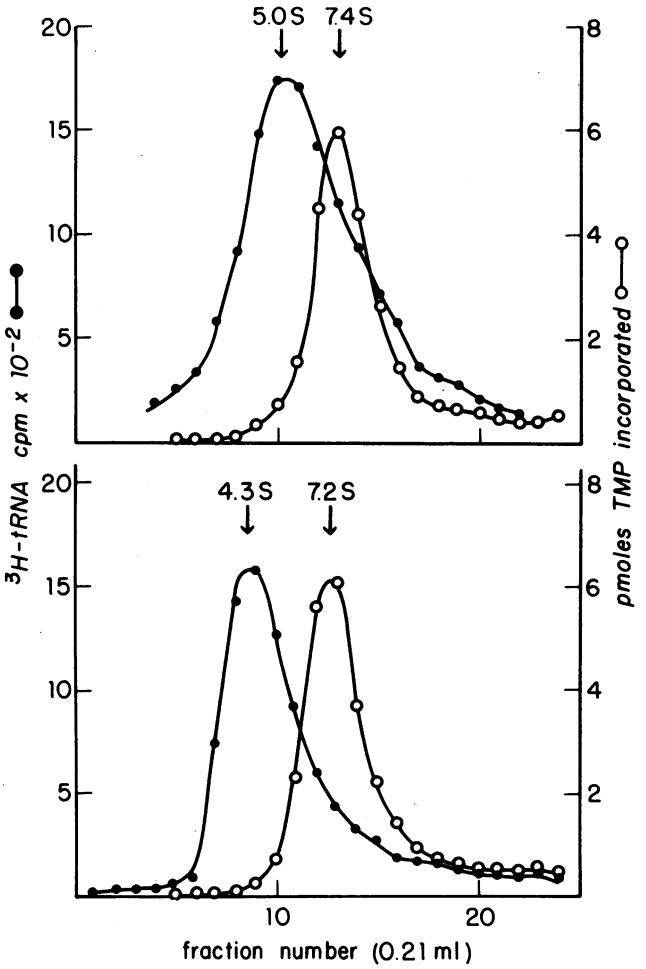

Figure 4. Sedimentation velocity in sucrose density gradients (10-30% w/v). The gradient buffer had the same composition as that used in the assays. The runs were carried out in an SW 50.1 rotor for 17 hours at 5° and 45,000 rpm. In the lower panel ^{3}H-tRNA (24,000 cpm, 0.025 M Tris, pH 7.4) and reverse transcriptase (7 units in 0.01 M Tris, pH 7.4) were layered (each in a volume of 100 λ) on top of separate tubes. ^{3}H-tRNA was determined by precipitating 100 λ of each fraction and processing it as described in Materials and Methods. Reverse transcriptase activity was monitored by assaying the activity of 10 λ of each fraction using poly rAdT$_{10}$ as the primer-template (see Materials and Methods). In the upper panel, ^{3}H-tRNA and reverse transcriptase were first mixed in 0.025 M Tris, pH 7.4, and 100 λ applied to the top of the gradient. ^{3}H-tRNA and reverse transcriptase activity were monitored as above.

side, so that it completely overlaps the region in which the reverse transcriptase sediments. This indicates that binding does occur in this buffer and that there is probably a rapid equilibrium among the components of the complex. However, the exact nature of the equilibrium in the different buffers is unknown and requires further investigation. Furthermore since all of the tRNAs of the sample contained tritium these data do not permit a determination of the extent of binding of each tRNA.

Interaction of E. coli tRNA with 70S RNA.

It was of importance to learn if the tRNAs could bind to AMV 70S RNA under assay conditions. Therefore the above velocity sedimentation experiment was repeated using 70S RNA with and without ^{3}H-tRNAs. The results are shown in Figure 5 where it can be seen that no measureable amount of radio-

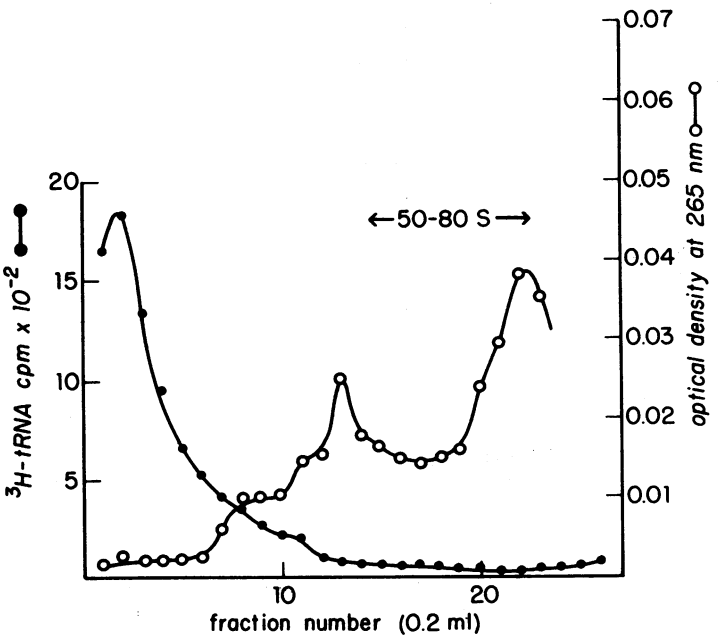

Figure 5. Sedimentation velocity in a sucrose density gradient (10-30% w/v). The gradient buffer had the same composition as that used in the assays. The curve on the left of the figure, ●——●, was obtained by sedimenting a mixture of E. coli ^{3}H-tRNA (about 24,000 cpm in 1.9 µg of tRNA) and 19.4 µg of AMV 70S RNA in 100 λ of 0.01 M Tris; the ^{3}H present in the tRNA was monitored. The curve on the right, o——o, represents 70S RNA only; in this case 38.8 µg were layered to be able to read the optical density directly. The run was carried out in an SW 50.1 rotor at 50,000 rpm for two hours at 5° C.

activity was found in the region 50-85S which contained the bulk of the 70S RNA, and we conclude that there is no appreciable binding of E. coli tRNAs to the viral RNA.

Interaction of tRNAs with 35S RNA.

When 70S RNA is heated it dissociates into 35S and 4S molecules (15,16). Annealing of this mixture leads to a recovery of about 80-90% of the original primer-template activity (8,17). We repeated this experiment in the presence of tRNAs and found that inhibition of the reaction occurred when the mixture was assayed in usual manner; the reaction mixture still contained the added tRNA. Inhibition was also observed when native 70S RNA was used with the same amount of tRNA in the assay mixture. This result implies that tRNA does not necessarily have to anneal to the primer location on the viral RNA, although it may, and that the observed inhibition is probably due to the binding of tRNA to the enzyme, as discussed above.

The effect of tRNAs on reverse transcription at low ionic strength.

We had shown earlier (10) that the sedimentation constant of AMV reverse transcriptase increases as the ionic strength of the sedimenting medium decreases. We also showed that the mechanism of reverse transcription was qualitatively different at low ionic strength. That is, the oligomeric forms of reverse transcriptase interact differently than does the monomer. We therefore tested the inhibitory effects of E. coli tRNAs at low ionic strength (0.06-0.08). We found that inhibition by tRNA was nearly negligible (data not shown) when the ratios of 70S RNA:tRNA were the same as those given in Figure 1. Therefore, oligomerization evidently results in the loss of binding sites for these tRNAs even though the oligomeric forms retain the $tRNA^{Trp}$ primer site(s) for the 70S AMV RNA.

DISCUSSION

We have studied the kinetics of reverse transcription by AMV reverse transcriptase using as template AMV RNA with its resident primer. We find that

addition of mixed E. coli tRNAs to the assay mixture results in inhibition of DNA synthesis. Significant inhibition occurs at a weight ratio of 70S RNA:tRNA of 10:1. $tRNA^{Phe}$ inhibits more efficiently than $tRNA_f^{Met}$ (data not shown). Whether the other tRNAs will show significant differences remains to be investigated. We have also shown that E. coli tRNAs which have been degraded to an $S_{20,w}$ value of 2.5 is much less effective as an inhibitor and that tRNA degraded to an $S_{20,w}$ value of 1.0 is ineffective (data not shown). These data tend to rule out that inhibition is due to a non-specific electrostatic effect. This question is under further investigation. We have also shown that yeast and mammalian tRNAs also inhibit synthesis (18). We find that the tRNA binds to the enzyme and not to the RNA, and we presume that the inhibition is due to the former interaction. We do not know whether there may also be a more complex interaction involving a 70S RNA-reverse transcriptase complex and tRNA.

It has been shown in other laboratories that $tRNA^{Trp}$ on the 70S viral RNA is an initiator of AMV reverse transcription in vitro (4-7), and that free $tRNA^{Trp}$ also binds specifically to the enzyme (19). In contrast to the results described here, a variety of other RNAs (e.g. yeast $tRNA^{Phe}$ and E. coli 4.5S RNAs) have been reported not to bind to AMV reverse transcriptase (19) on the basis of experiments involving G-100 Sephadex gel filtration. Our results, however, were obtained using sucrose density gradient velocity centrifugation. The difference probably lies in the techniques used; the Sephadex gel matrix probably disrupts the equilibrium between tRNA and reverse transcriptase by separating the two components.

A word regarding the nature of the inhibition by added tRNAs is in order. At low ionic strengths (0.06-0.08) where reverse transcriptase exists as an oligomer of the 160,000 $\alpha\beta$ molecule (10), DNA synthesis is optimal, yet we observe no inhibition by added tRNAs. Therefore the $tRNA^{Trp}$ and any other essential sites on the enzyme must be available, and we may deduce that inhibition by tRNAs does not involve a direct competition for these specific

sites on the enzyme. A variety of explanations for the phenomenon are possible. One is that the added tRNA may act as a negative effector through an allosteric mechanism, i.e. the active site may be altered by interaction of the effector tRNA at a site on the enzyme which is not accessible in the oligomer. We have presented evidence for allosteric effects in the E. coli Pol I system (20). Other possibilities involving classical non-competitive or uncompetitive mechanisms are now under investigation.

The ability of a natural substance, tRNA, to inhibit reverse transcription in vitro is profoundly interesting and evokes speculation regarding the in vivo control of this type of synthesis, the reverse flow of information (21). Cellular tRNAs, most of which are irrelevant as primers for viral RNA, may serve to prevent reverse transcription by a mechanism similar to that occurring in the in vitro system (18). Therefore we are examining the inhibitory effects of a number of non-virion tRNAs in various types of in vivo systems (11).

REFERENCES

1 Verma, I.M., Meuth, N.L., Bromfeld, E., Manly, K.F. and Baltimore, D. (1971) Nature New Biol. 233, 131-134
2 Dahlberg, J.E., Sawyer, R.C., Taylor, J.M., Faras, A.J., Levinson, W.E., Goodman, H.M. and Bishop, J.M. (1974) J. Virol. 13, 1126-1133
3 Faras, A.J., Dahlberg, J.E., Sawyer, R.C., Harada, F., Taylor, J.M., Levinson, W.E., Bishop, J.M. and Goodman, H.M. (1974) J. Virol. 13, 1134-1142
4 Ikemura, T. and Dahlberg, J.E. (1973) J. Biol. Chem. 248, 5024-5032
5 Sawyer, R.C. and Dahlberg, J.E. (1973) J. Virol. 12, 1226-1237
6 Sawyer, R.C., Harada, F. and Dahlberg, J.E. (1974) J. Virol. 13, 1302-1311
7 Harada, F., Sawyer, R.C. and Dahlberg, J.E. (1975) J. Biol. Chem. 250, 3487-3497
8 Waters, L.C., Mullin, B.C., Ho, T. and Wen-Kuang, Y. (1975) Proc. Natl. Acad. Sci. USA 72, 2155-2159
9 Cavalieri, L.F. (1974) J. Virol. 14, 1458-1462
10 Yamaura, I. and Cavalieri, L.F. (1975) J. Virol., submitted for publication
11 This work is being done in collaboration with Dr. H. Hanafusa
12 Kacian, D.L., and Spiegelman, S. (1973) Methods in Enzymology Vol 29E, 150-173, Academic Press Inc. N.Y.
13 Kacian, D.L., Watson, K.F., Burny, A. and Spiegelman, S. (1971) Biochim. Biophys. Acta 246, 365-383
14 McEwen, C. (1967) Anal. Biochem. 20, 114-119
15 Canaani, E. and Duesberg, P.H. (1972) J. Virol. 10, 23-31
16 Dahlberg, J.E., Sawyer, R.C., Taylor, J.M., Faras, A.J., Levinson, W.E., Goodman, H.M. and Bishop, J.M. (1974) J. Virol. 13, 1126-1133
17 Faras, A.J. and Dibble, N.A. (1975) Proc. Natl. Acad. Sci. USA 72, 859-863
18 Yamaura, I. and Cavalieri, L.F. (1975) Nature, submitted for publication

19 Panet, A., Haseltine, W., Baltimore, D., Peters, G., Harada, F. and Dahlberg, J. (1975) Proc. Natl. Acad. Sci. USA 72, 2535-2539
20 Cavalieri, L.F., Modak, M.J. and Marcus, S.L. (1974) Proc. Natl. Acad. Sci. USA 71, 858-862
21 Lee Huang, S. and Cavalieri, L.F. (1963) Proc. Natl. Acad. Sci. USA 50, 1116